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CYSTINOSIS (LIGNAC-FANCONI DISEASE)*

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Cystinosis is a relatively uncommon disease of infants and children characterized by retarded physical development which results in dwarfism, storage of cystine crystals in the cells of the reticulo-endothelial system, and amino-aciduria. Severe rickets usually is present, but may be absent. Lignac's[1] classical description of the disease in 1924 has resulted in the synonymous terms cystine storage disease, cystinosis, and Lignac's disease.

Because of the similarity between Fanconi's cases[2-4] and cystinosis, Bickel[5] recently has proposed that the term Lignac-Fanconi disease be used. Owing to Fanconi's continued interest in, and study of,[6] this condition, it appears to be well known in Europe. His association with Bickel[7] has, at least in part, been responsible for the extensive studies recently reported from England.[5,8-15] On the other hand, on this side of the Atlantic, the characteristic hexagonal crystals have been seen rarely.

The first report of cystinosis in the American literature was of 2 cases[16] in 1950. One case was reported by King and Lochridge[17] in 1951. The following year, 2 cases were reported by Guild, Walsh, and Hoover[18] in which the diagnosis was made by slit lamp examination of the cornea and the presence of characteristic crystals in the bone marrow.

REPORT OF CASE

Present Illness. A white female child, 33 months old, was admitted on January 18, 1954, because of dehydration and acidosis. One week previously she had been treated with penicillin for an infection of the upper respiratory tract characterized by cough and a temperature of 104° F. At that time roentgenograms of the wrists were diag-

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nostic of rickets. Oleum percomorphum, Roncovite, and vitamin mixture were prescribed. Three days prior to admission she refused food and liquids and the day before admission she had respiratory distress which consisted of rapid respirations followed by short intervals of apnea. The patient was admitted from another hospital where laboratory studies revealed the non-protein nitrogen of the blood to be 31 mg. per 100 ml. and the carbon-dioxide combining power, 13.8 volumes per cent.

Past History. The birth weight was 5 lbs., 15 oz., following a full-term, normal pregnancy. The neonatal period was uneventful and the patient took an evaporated milk formula well. She had "routine" immunizations at 6 months. Baby foods were started at 6 months and Zymogen drops at 9 months. The appetite was always poor. She chewed her food very slowly and had frequent episodes of regurgitation and vomiting. She ate meat only occasionally, refused fruits, and had a craving for liquids and salt. She was especially fond of salty potato chips. At 8 months of age she was transfused with whole blood because of anemia. Her motor development was very poor; she sat up at 8 months and never crawled or walked. She was able to get around by holding onto chairs and tables. Her mental development seemed normal and, although her speech was delayed, she had been using words in sentence form during the last 6 months of life.

Family History. A sister died in 1939 at the age of 30 months with terminal illness of vomiting, diarrhea, convulsions, and a rectal temperature of 108° F. She was anemic and had repeated episodes of vomiting and upper respiratory tract infections. The physical development of this sister was retarded and she had just begun to walk before her terminal illness. The mother described this child as being similar to the patient in "looks and actions."

Physical Examination. On examination, the temperature was 103.2° F.; pulse, 104; weight, 14 lbs., 6 oz.; length, 29 inches. The patient was a pale, underdeveloped, malnourished, moribund white female child with gasping respirations. The skin was dry and there was a loss of turgor. The anterior fontanel was open 0.5 cm. and depressed. The eyes, ears, and nose were normal. The mouth contained mucoid secretions and the lips were dry and crusted. The neck was supple. The thorax was symmetric and there was enlargement of the costochondral junctions. Breath sounds were bronchial in nature and the respirations were irregular and gasping. The heart was normal. No abdominal organs or masses were palpable. The muscle tone of the extremities was poor. There was enlargement of the wrists, elbows, ankles, and knees. Neurologic examination showed areflexia. Shortly after the physical examination, the patient died.

Post-mortem examination was performed 3 hours after death and after roentgenograms of the extremities were made.

Gross Examination

The external findings were similar to those described in the physical examination. The thymus weighed 3 gm. It was soft and light pink. The thyroid gland weighed 4 gm. There were several small lymph nodes along the lateral aspect of each lobe. Only one parathyroid gland was identified. It was enlarged, measuring 5 by 3 mm. There was no free fluid present in the serous cavities. The lungs weighed 60 gm. They were light pink, soft, and crepitant, with small foci of atelectasis in the dependent portions. The tracheobronchial tree was normal. The heart weighed 40 gm. and was normal. The abdominal

viscera were all in their proper locations. The liver weighed 240 gm. It was pale brown and smooth. On cut section, no abnormalities were noted. The gallbladder was normal. The extrahepatic biliary ducts were patent. There was lymphoid hyperplasia of the small intestine and Peyer's patches were prominent. There was pallor of the gastrointestinal tract. There was moderate enlargement of the lymph nodes in the mesentery of the small and large intestine. The enlarged nodes were discrete and pale gray, and on cut section there was slight bulging with numerous chalky, yellow-gray, linear streaks. The spleen weighed 23 gm. It was red-gray and soft. On cut section, there were numerous small, yellowish, chalky, linear streaks. The splenic pulp and the chalky material did not scrape easily. The pancreas weighed 18 gm., was firm, gray-pink, and normal. The adrenal glands together weighed 3.5 gm. The kidneys each weighed 45 gm. They were pale, and the surface was slightly granular. The capsule stripped easily. On cut section, there was good corticomedullary differentiation. There was a constriction at the ureteropelvic junction on the right side but no true obstruction was present. The right ureter was slightly dilated, but the pelvis of the right kidney was normal. The internal genitalia were normal. The bone marrow from the vertebral column was light brown. The brain was normal and weighed 880 gm. The meninges were normal.

Post-mortem roentgenograms were interpreted by Dr. J. A. Campbell as follows: "There is evidence of a severe generalized skeletal growth disturbance characterized by hypocalcification and advanced rachitic changes of a type suggesting renal insufficiency."

Microscopic Examination

Hematoxylin and Eosin-stained Sections

Mesenteric Lymph Nodes. The architecture of the lymph nodes was somewhat altered by the reduced size of the cortical follicles and the prominence of the medullary cords as well as obliteration of the sinuses by reticulo-endothelial hyperplasia. There were numerous large vacuolated cells lining the sinusoids and arranged in nests and linear strands in the medullary cords. These cells had a distinct cellular membrane, a round, dark, eccentrically placed nucleus and scant, coarsely reticulated cytoplasm. Further study of unstained material indicated that these vacuolated cells had contained crystals of cystine. There was slight reticular hyperplasia of the cortical follicles, some of the reticulum cells showing vacuolated cytoplasm. The peripheral sinuses were distended and contained numerous lymphocytes.

Spleen. In the spleen there was a diminution in both the number and size of the lymphoid follicles, most of which showed slight reticular hyperplasia. The central arterioles were made conspicuous by the presence of a cuff of vacuolated cells, one or two cells thick. The cords of Billroth were widened by sheets of large vacuolated cells. Vacuolated cells were present around the trabeculae and trabecular arteries.

Liver. In the central portion of the hepatic lobule there was slight atrophy of the cord cells and separation of the sinusoidal membrane from the parenchymal cells. There were large, round and oval, vacuolated cells with eccentric nuclei. These cells measured 10 to 25 μ in diameter. They were present singly and in small clusters and were located between the hepatic cells and the sinusoidal membrane, particularly in the central portion of the lobule. Occasionally, clusters of two or three such cells were seen on either side of a sinusoid which was compressed.

Kidneys. The renal capsule was slightly thickened. There was diffuse interstitial fibrosis with lymphocytic and mononuclear infiltration, especially in the peripheral portion of the cortex. Slight interstitial edema was present. Scattered throughout the interstitial tissues were occasional large vacuolated cells. The majority of the glomeruli were large and each filled Bowman's space. There was proliferation of the endothelial cells of the glomerular tufts and thickening of the capillary basement membranes. The parietal layer of Bowman's capsule was prominent, showed hyaline thickening and an increased cellularity, with an occasional epithelial crescent present. Other glomeruli were small and showed moderate thickening of the basement membrane. An occasional fibrosed glomerulus was seen. The convoluted tubules in the cortex were irregularly dilated and lined by cuboidal or flattened epithelium. Many of the epithelial cells contained a large, clear vacuole which displaced the nucleus laterally and basally and caused the cell border to protrude into the lumen. Some of the vacuoles appeared to occupy the entire cell. In the basilar regions of many of the epithelial cells which did not have large vacuoles, there were numerous small vacuoles which contained lipid. There was slight granular degeneration of the cytoplasm of the epithelial cells. The nuclei of the vacuolated cells were normal in appearance but there were occasional pyknotic nuclei in the flat, non-vacuolated epithelial cells which showed advanced degrees of granular degeneration. The majority of the tubules were filled with the remains of exfoliated epithelial lining cells which had undergone hydropic degeneration. Some of the tubules also con-

tained a small amount of eosinophilic amorphous granular material. The arterioles showed moderate hyaline thickening of the walls. The renal pelvis was not unusual.

Lungs. The lungs showed focal areas of bronchopneumonia characterized by a polymorphonuclear exudate in the bronchi and surrounding alveoli.

Cardiovascular System. The medium-sized mesenteric vessels showed subintimal thickening which consisted of a homogeneous, slightly eosinophilic, hyalin-like material. This did not stain with crystal violet, and with the Mallory trichrome stain it appeared light blue.

Bone (Rib). The rib showed irregular widening of the zone of proliferation and maturation. The normal columnar arrangement of the proliferating cartilage cells was maintained only in that portion adjacent to the resting cartilage. There was no zone of provisional calcification of cartilage and the vascular fibrous connective tissue of the marrow spaces invaded the zone of maturation irregularly. The wide and irregular osteoid trabeculae were poorly calcified. There was fibrosis of the bone marrow immediately surrounding the trabeculae as well as lacunae of loose fibrous connective tissue within the trabeculae, especially near the metaphysis. The erythropoietic elements were decreased. Only an occasional fat cell was seen. Scattered throughout the bone marrow were many large vacuolated cells. These were most numerous immediately surrounding the trabeculae.

Parathyroid Glands. The cells of the parathyroid gland were more densely packed than usual. There was a noticeable absence of fat cells. Chief cells with clear cytoplasm predominated. They were only slightly enlarged. No oxyphilic cells were present.

Pancreas. Some of the pancreatic acini were dilated and contained eosinophilic inspissated secretions. There was no fibrosis.

Thymus. The thymic cortex was thin and the medulla contained vacuolated cells.

Brain. Sections of the brain were normal except for the choroid plexus which showed numerous vacuolated cells in the stroma.

Gastrointestinal Tract. The gastrointestinal lymphoid tissue was hyperplastic. The centers of the follicles contained vacuolated cells. There were vacuolated cells in the tunica propria of the mucosa of the ileum.

Sections of the heart, esophagus, stomach, duodenum, colon, gallbladder, adrenal gland, urinary bladder, fallopian tubes, ovary, uterus, vagina, thyroid gland, tonsil, skeletal muscle, aorta, and trachea were normal.

Demonstration of Cystine Crystals in the Tissues (Figs. 2 to 5)

Although cystine is soluble in formalin and other aqueous fixatives, cystine crystals could still be seen in tissues which had been fixed in formalin or Helly's fluid for 48 hours. The usual technique for hematoxylin and eosin staining dissolves the crystals; therefore, paraffin or frozen sections were examined unstained or stained with alcoholic methylene blue.

Mesenteric Lymph Nodes. The mesenteric lymph nodes showed many round and oval clumps of densely packed crystals, of which the individual outlines could not be distinguished. These clumps measured up to 40 μ in diameter and were sharply outlined. In an occasional clump a nucleus was visible, indicating that these crystals were intracellular. The clumps of crystals were grouped in aggregates of thirty to forty, primarily in the medulla and along the medullary cords of the lymph nodes. Two or three clumps of crystals were present in the center of every lymph follicle. Clumps of crystals were seen around the blood vessels and along trabeculae. Scattered throughout the stroma were numerous solitary crystals. These crystals were small, flat, and hexagonal, rectangular, or fragmented. When viewed under polarized light, they were birefringent.

Spleen. Cystine was deposited in the reticulo-endothelial cells of the spleen, primarily along the cords of Billroth in the red pulp. An occasional cluster of crystals was seen in the lymphoid follicles.

Liver. Cystine crystals in aggregates of two or three clumps were found between hepatic cords, primarily near the central region of the lobules.

Bone Marrow. Clumps of crystals were seen throughout the bone marrow, but they were especially numerous in the region adjacent to the trabeculae.

Thymus. Crystals were present in the medulla of the thymus in an irregular fashion, no definite architectural pattern being discernible.

Intestinal Lymphoid Tissue. Only an occasional clump of cystine crystals was present in the center of the submucosal lymphoid follicles of the intestine.

Choroid Plexus. Numerous clumps of cystine crystals were present in the stroma of the choroid plexus.

Cystine crystals were seen singly and in small clusters in the interstitial tissues of the pancreas, thyroid gland, adrenal capsule, lung, and mucosa of the ileum.

Isolation and Chemical Identification of Cystine

Following the method of King and Lochridge,[17] crystalline cystine was isolated from alcohol-fixed mesenteric lymph nodes which had been fixed previously in 10 per cent formalin for 1 week. Microscopically, this crystalline material was composed of hexagonal plates and fragments of hexagonal plates, with occasional rectangular forms. The following tests were performed on the isolated crystals: (1) Upon the addition of strong HCl, cystine hydrochloride crystals were formed. These were characteristically long, needle-shaped crystals which occurred singly and in clusters. (2) Upon the addition of phosphotungstic or phosphomolybdic acid as described by Bullock and Kirk,[19] typical crystals of cystine phosphotungstate and cystine phosphomolybdate were formed. (3) The test for labile sulfur was strongly positive. (4) The melting point was determined to be 260° with disintegration at 261° C. (5) After treatment with 5 per cent sodium cyanide, the nitroprusside reaction was strongly positive. The nitroprusside reaction was negative without treatment with sodium cyanide. (6) Sullivan's test,[20] which is specific for cysteine or cystine, was strongly positive.

Because Sullivan's test[20] was strongly positive, it was decided to determine the content of cystine by photocolorimetry. The crystalline material isolated from 26 mg. (dry weight) of alcohol-fixed mesenteric lymph nodes was dissolved in 0.1 N HCl. Sullivan's test was done on this solution and on a standard solution of l-cystine in 0.1 N HCl. The two solutions were compared on the photocolorimeter and it was determined that 1.38 mg. or 5.3 per cent of cystine was present in the dried tissue.

Paper electrophoretic studies on material isolated from the mesenteric lymph nodes indicated that only one substance was present in the extract and that this substance migrated with l-cystine.

Discussion

Following their detailed clinical study of 14 cases of Lignac-Fanconi disease, Bickel *et al.*[9] tabulated the clinical and chemical features on which the diagnosis of this condition rests. This table is reproduced on the following page* with the features found in the present case indicated by the use of italics.

* Reproduced with permission of Dr. H. Bickel, and Dr. B. Vahlquist, Co-Editor of *Acta Paediatrica*.

A. Characteristic clinical findings.
   (a) *Dwarfing.*
   (b) *Cystine storage in eyes and bone marrow.*
   (c) Photophobia.
   (d) *Resistant rickets, bony deformities, pathological fractures.*

B. Non-specific clinical findings.
   (a) *Thirst, polyuria.*
   (b) *Failure to thrive and to gain weight.*
   (c) *Anorexia and attacks of vomiting.*
   (d) *Susceptibility to infection, unexplained fever, metabolic crises.*
   (e) *Muscular weakness, delayed standing and walking.*
   (f) *Onset between 6 and 12 months of age, but later in the chronic form.*
   (g) *Affection of other siblings.*
   (h) Latent and manifest tetany in the late stages.

C. Chemical findings (none of which are constant).
   (a) Albuminuria, polyuria, scanty casts and cellular elements in the deposit.
   (b) Variable aminoaciduria and glycosuria.
   (c) *Bicarbonate low in plasma, high in urine with alkaline urine.*
   (d) Plasma phosphorus low or raised. Phosphatase normal or raised.
   (e) Plasma potassium low, sodium and chloride lowered slightly.
   (f) Cholesterol normal or raised.

In retrospect, the clinical features in the present case are rather typical and indeed the diagnosis was suspected while one of us (R.L.N.) was treating the patient for recurrent episodes of fever with respiratory infections. Hospitalization was not feasible until the final episode. The urines of 8 immediate members of the family were examined and none showed the presence of sugar, albumin, or cystine by chemical test (Sullivan's test[20]). Recent studies suggest that the disease is genetically determined and of simple mendelian recessive character.[8]

Once suspected, the diagnosis may be established by finding cystine deposits in the cornea by use of the slit lamp.[12,18,21,22] Biopsy of the conjunctiva may reveal the presence of cystine crystals.[12,22] In the

presence of photophobia there are dystrophic changes in the corneal epithelium, without ulceration, detectible by fluorescein.[12] Bone marrow aspiration will reveal typical crystals[18,23] but they may be very scarce as many of the crystals dissolve in the process of staining. Unstained films of the bone marrow should reveal an abundance of crystals. Biopsy of a lymph node will reveal the diagnosis if the proper staining procedures are used.[24]

Urine studies by paper partition chromatography characteristically reveal an amino-aciduria[7,9,25,26] which, with some variation in the number and kind of amino acids, also has been reported as a finding in patients with uncomplicated rickets.[27] Rickets occasionally may be absent[17,28] or very mild,[6] but usually is present in a very severe form which has been intractible and resistant to vitamin D therapy.

The degree of renal involvement is highly variable. The kidneys may be normal[29,30] or they may show interstitial fibrosis, tubular atrophy, and degeneration.[17,31] Glomerular changes, varying from slight focal necrosis to complete fibrosis and hyalinization,[15] usually are associated with diffuse interstitial fibrosis, tubular degeneration, and arteriolar sclerosis. Hydropic degeneration of the tubular epithelium,[15,32] associated with glomerular changes with the exception of one case,[17] bears a striking similarity to the tubular changes described by Kulka, Pearson, and Robbins[33] in 15 patients with intestinal obstruction who also had water and electrolyte disturbances.

Enlargement of the parathyroid glands, usually associated with rickets and advanced renal lesions, sometimes is found. The cellular architecture may be normal[28] or there may be a predominance of water-clear chief cells.[15]

Summary

A case of cystinosis characterized by the presence of cystine crystals in the cells of the reticulo-endothelial system, rickets, and dwarfism is reported. Renal changes consisted of glomerular sclerosis, interstitial fibrosis, arteriolar sclerosis, and hydropic degeneration of the tubular epithelium. Cystine, isolated from mesenteric lymph nodes, was identified by several chemical tests.

The chemical isolation and quantitative determination of cystine were done by Dr. H. R. Onyett. Photomicrographs are by Messrs. James F. Glore and Paris Johnson of the Department of Illustration of the Indiana University Medical Center.

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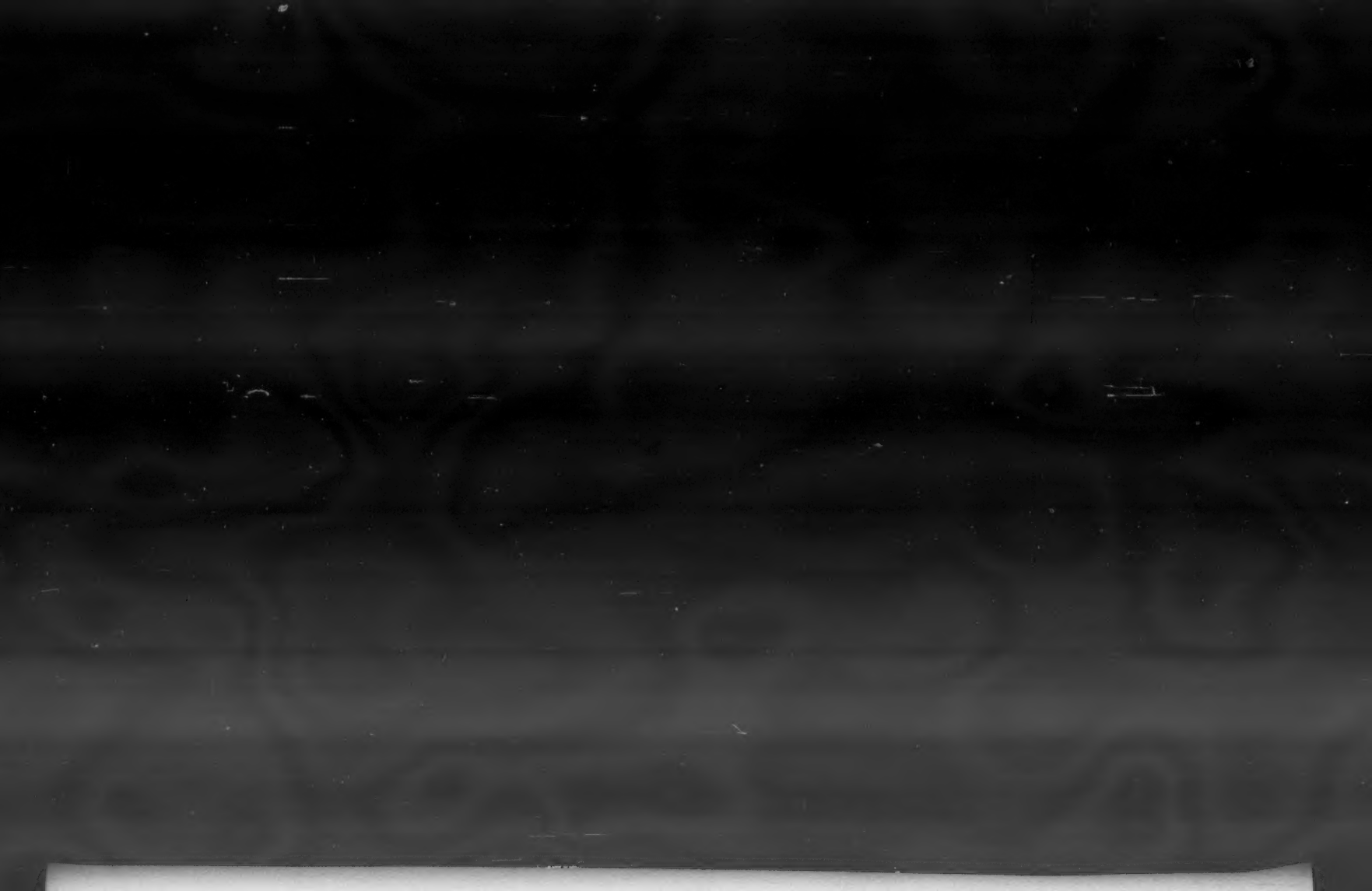
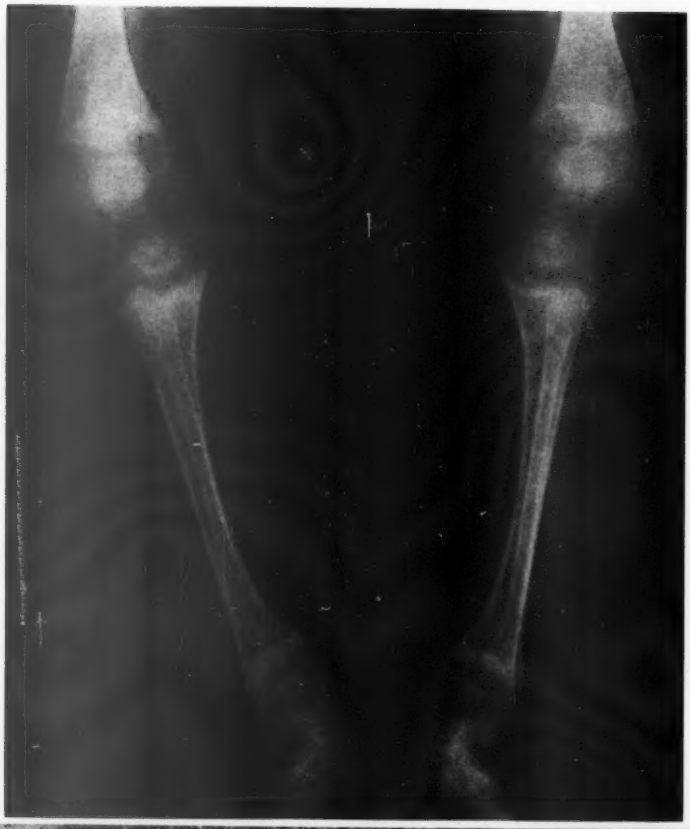
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Reproduction of a post-mortem roentgenogram, showing advanced rachitic changes of the lower extremities.

FIG. 2. Alcohol-fixed mesenteric lymph node stained with alcoholic methylene blue. Clumps of cystine crystals are present in aggregates and columns. Solitary crystals are scattered throughout the section. × 150.





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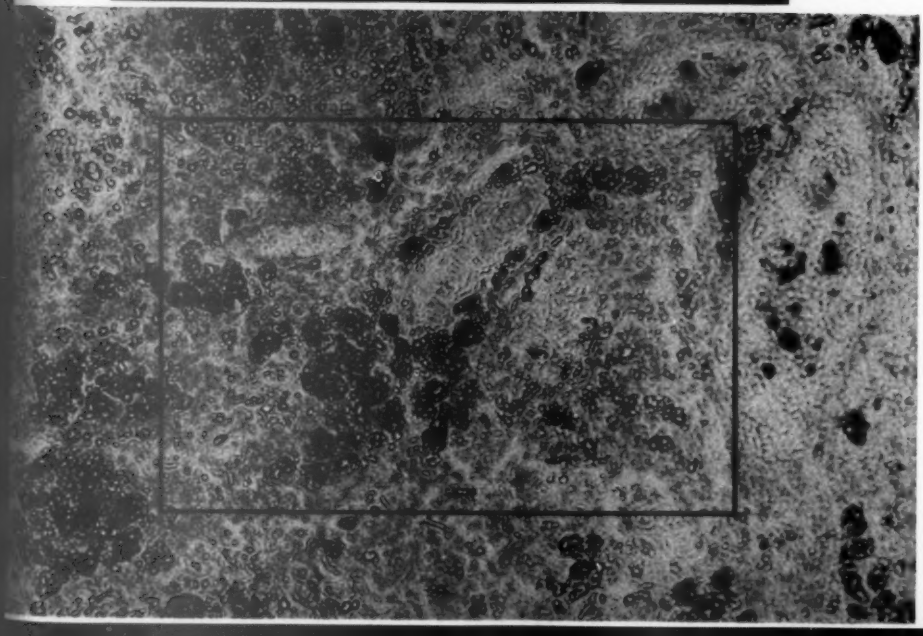

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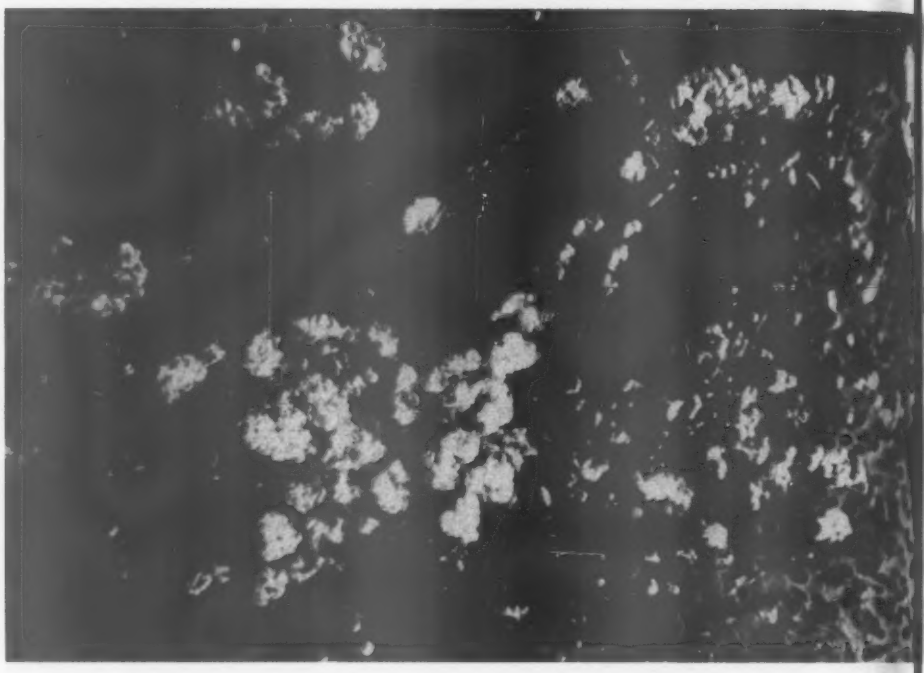
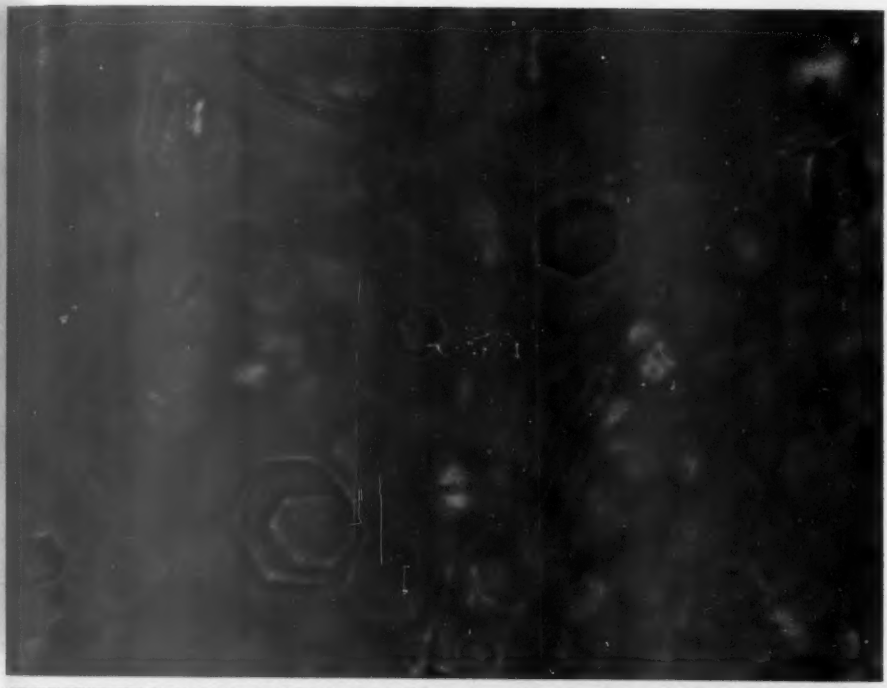


FIG. 3. Higher magnification of the boxed area of Figure 2 with modified dark-field illumination. Individual clumps of cystine crystals are well outlined, implying intracellular location. × 250.

FIG. 4. Unstained frozen section of alcohol-fixed mesenteric lymph node. Flat hexagonal crystals, characteristic of cystine, are scattered extracellularly. × 800.

FIG. 5. Crystals of cystine obtained from aqueous extract of alcohol-fixed mesenteric lymph nodes. × 935.



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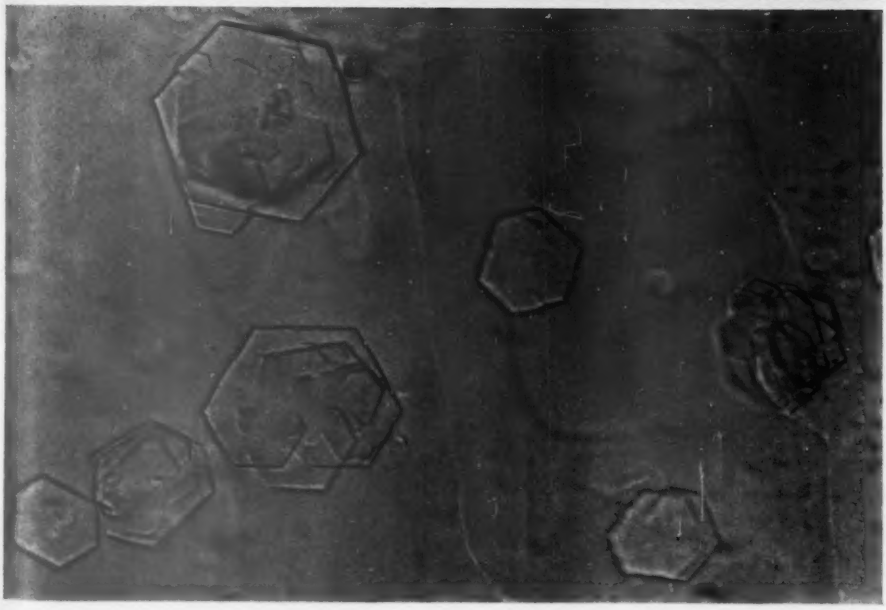

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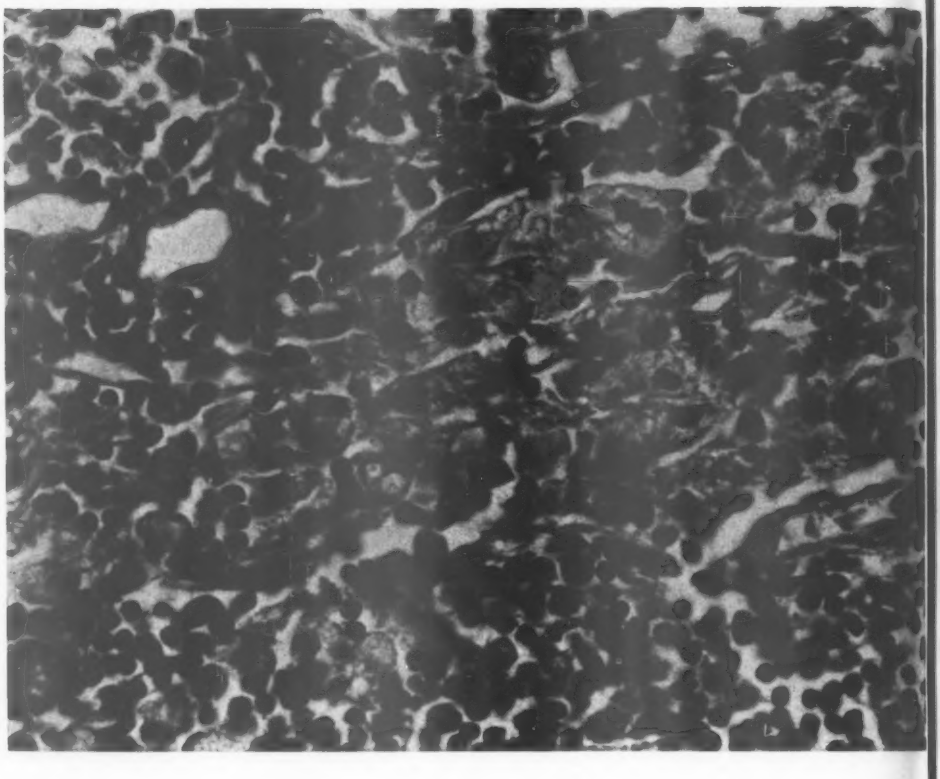
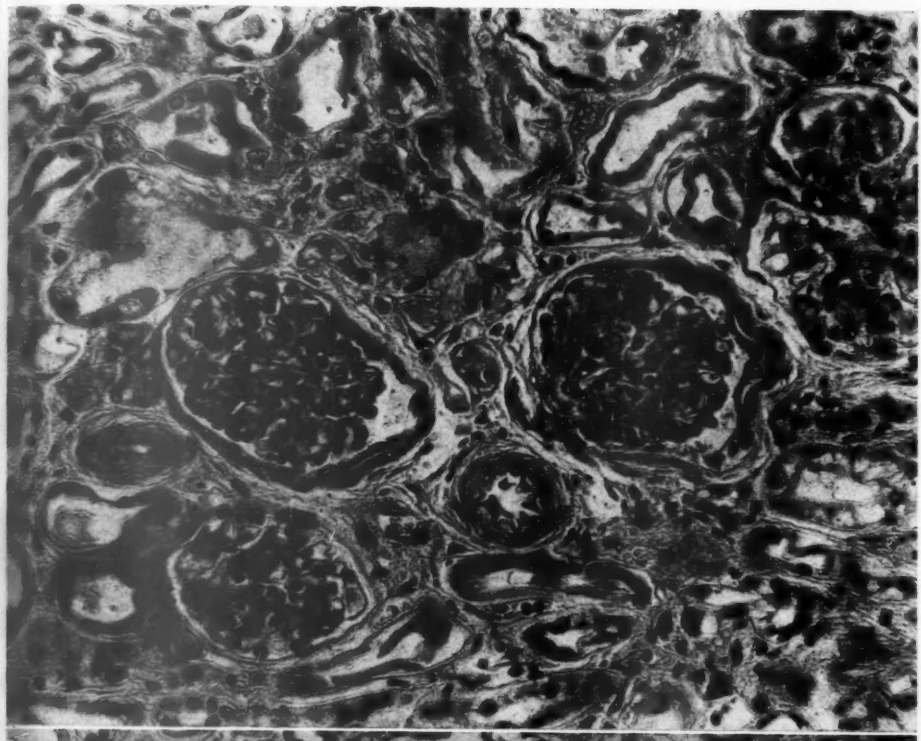


FIG. 6. Masson's trichrome stain of alcohol-fixed mesenteric lymph node showing a group of large vacuolated reticulo-endothelial cells. × 540.

FIG. 7. Hematoxylin and eosin stain of formalin-fixed kidney. The parietal layer of Bowman's membrane is prominent and the arterioles are thickened. × 260.

FIG. 8. Hematoxylin and eosin stain of formalin-fixed kidney. The tubules are irregularly dilated and the epithelium shows vacuolar degeneration. × 260.



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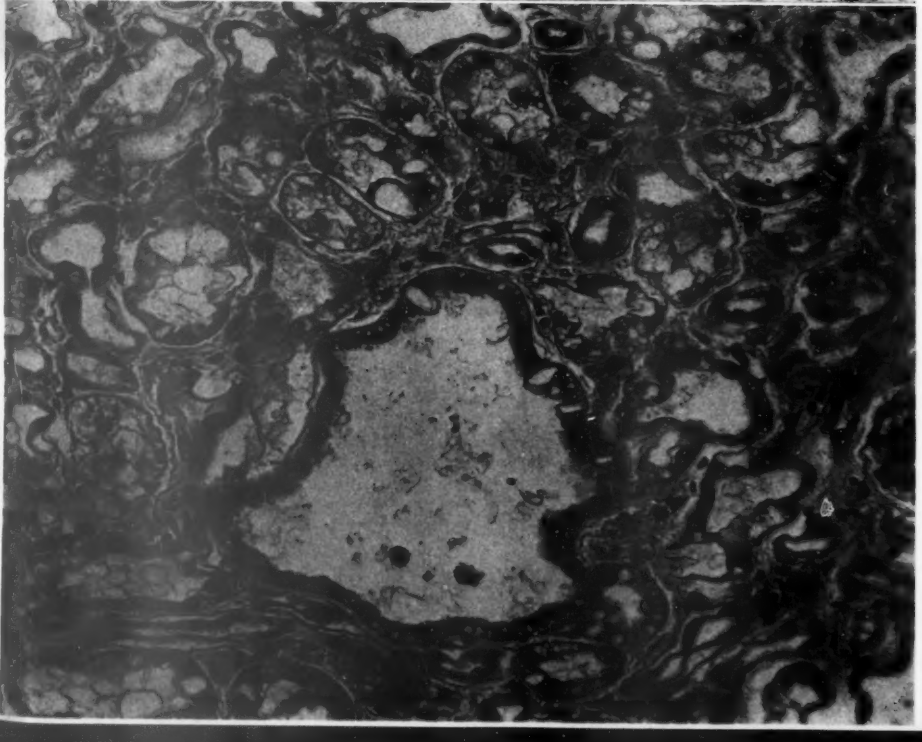

8

RHEUMATIC ACTIVITY IN AURICULAR APPENDAGES REMOVED AT MITRAL VALVOPLASTY *

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Seventy-eight auricular appendages from patients with progressive cardiac failure presumably of rheumatic origin were available from valvoplasty procedures performed by Dr. W. Bigelow, of the Department of Surgery, Toronto General Hospital. Since the most recent operation in this particular group was carried out at least 18 months ago, sufficient time has elapsed to report on the incidence of recrudescence, and on any relationship which might exist between the reappearance of clinical rheumatic fever and the extent and nature of the auricular lesions encountered. Although a voluminous literature is rapidly accumulating on the subject of rheumatic activity in auricular appendages, the relatively high incidence of myocardial granulomas and a peculiar basophilic alteration of the connective tissues of both myocardium and endocardium have not hitherto been emphasized.

Material

Included in the material were routine surgical sections from 78 left auricular appendages, together with the appendages from 25 necropsies on non-rheumatic subjects of the same age group. The patient age varied from 17 to 53 years, the average being 35.8 years. The control appendages showed no changes other than varying degrees of thickening of the endocardium.

Incidental to the study of the heart material were 29 specimens of lung taken for biopsy. They were examined for parenchymal and vascular changes which might indicate chronic pulmonary hypertension. Fourteen showed slight to moderate degrees of fibrous thickening of alveolar walls, while in another 4 cases the changes were fairly marked. Medial hypertrophy and/or fibrosis was present in small arteries and arterioles in approximately one half of the specimens of lung. Some showed evidence of intimal fibrosis as well. In only 2 cases were vascular changes present without evidence of fibrotic changes in the parenchyma. Larrabee, Parker, and Edwards[1] reported a series of 20 cases in which vascular changes were present in 75 per cent. Heart lesion cells were present in 21 cases, sometimes in considerable numbers.

* Received for publication, December 30, 1954.

Lesions of Auricular Appendages

The auricular lesions included endocardial and myocardial granulomas, basophilia of connective tissues, fibrosis, and cellular infiltration. Recent and old intra-auricular thrombi were present frequently.

Granulomatous lesions were encountered in both endocardium and myocardium (Table I). They varied in shape from round to elliptic, and their site of predilection was between myocardium and endocardium, or in the deeper layers of the latter. They were occasionally so numerous as to form an almost continuous band (Fig. 1). The relatively small number found in myocardium (Fig. 2) had usually a perivascular distribution. The lesions were largely characteristic Aschoff nodules (Figs. 3 and 4), with a central nidus of fragmented, swollen, collagen fibers. Within the loose connective tissue were numerous large, darkly staining, mononuclear and multinucleated Aschoff cells, often of bizarre shape and with pale or dark nuclei. Small numbers of round cells and very occasional polymorphonuclear leukocytes and plasma cells were present in the lesions. Other lesions were devoid of multinucleated Aschoff cells, and contained numerous, fairly large, elongated, mononuclear cells with dark cytoplasm and nucleus. Some of the older lesions did not show fibrinoid or basophilic change, and contained only a few compressed fibers throughout their length. In a few cases, areas of rather dense acellular connective tissue, having a somewhat concentric appearance, may have represented healed lesions. The collagen fibers in and around the granulomatous foci frequently showed a basophilic tinctorial change, and the latter was found also throughout the connective tissues uninvolved by granulomas. Fibrinoid change was most marked in relation to the Aschoff lesions, although occasional collagen fibers in the deep layers of endocardium showed similar alteration.

Table I

Incidence and Severity of Lesions in 78 Auricular Appendages

	Granulomas				Basophilic degeneration				Cellular infiltration				Fibrosis			
	Endocardial		Myocardial		Endocardial		Myocardial		Endocardial		Myocardial		Endocardial		Myocardial	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Mild	31	39.7	6	7.7	29	37.2	19	24.4	34	43.6	14	17.9	25	32.1	7	8.9
Moderate	5	6.4	1	1.3	6	7.7	4	5.1	1	1.3	1	1.3	3	3.9	0	.0
Severe	3	3.9	0	.0	6	7.7	3	3.9	0	.0	0	.0	0	.0	0	.0
Total	39	50	7	9	41	52.6	26	33.4	35	44.9	15	19.2	28	36.0	7	8.9

Basophilic degeneration of connective tissue was the most common finding. The myocardial interstitial connective tissue and endocardium

were involved, sometimes extensively. This altered tissue stained metachromatically with toluidine blue. The fibers occasionally were swollen, or thin and frayed. The muscle fibers often were more widely separated than usual, due to abundant loose basophilic connective tissue (Fig. 6). The basophilic alteration frequently occurred independently of either Aschoff granulomas or fibrinoid degeneration. Fibrous endocardial thickening was common and tended to be irregular in distribution. The control material showed a fairly wide variation in this respect and it was difficult to assess minor degrees of endocardial thickening. Lymphocytic infiltration often was observed in endocardium and myocardium, and occasionally was accompanied by small numbers of polymorphonuclear leukocytes and plasma cells. The cellular infiltration usually was associated with granulomas, intra-auricular thrombi, or endocardial thickening.

Only 10 of the 78 appendages did not show one or more of the lesions, although this is not indicated in Table I. The changes were graded as mild, moderate, or severe. Sections containing only one or two granulomas were listed as showing mild changes, those with eight or ten or more, as severe, the intermediate grade being grouped as moderate.

Evidence of old intra-auricular thrombosis (Figs. 5 and 7) was present in 25.6 per cent of cases and of recent thrombosis in 20.5 per cent. Of these, 3 cases, or 3.9 per cent, showed both recent and old thrombi.

Myocardial granulomas never were present without endocardial granulomas, and in only one case was myocardial basophilic change present without similar degeneration in the endocardium. The incidence of endocardial Aschoff lesions (50 per cent) approximated fairly closely the figure of 45.3 per cent reported by Decker *et al.*[2] and by McNeely *et al.*[3] in a series of 183 cases. These authors reported no truly myocardial lesions, although these were present in a perivascular distribution in 9 per cent of the series here reported. Enticknap[4] reported granulomas in 41 per cent of a series of 71 cases and McKeown[5] found them in 45 per cent of 53 biopsies. However, Janton *et al.*[6] found an incidence of only 13 per cent in 88 cases.

Interrelationship of Lesions

The most common change was basophilic degeneration of connective tissue. This was present without accompanying granulomas in 19 cases (24.4 per cent). Conversely, in 16 cases (20.5 per cent), Aschoff lesions were present without basophilic change outside the granulomas. The two were associated in 23 cases (29.5 per cent). The Aschoff

lesions were found with endocardial or myocardial fibrosis in 16 cases, although in only 2 of these was fibrosis found alone with granulomas. In 12 cases, the latter were associated with thrombi.

Basophilic degeneration accompanied fibrosis in 15 cases, and the latter change was associated with thrombi in 16 cases. The degeneration was present with recent or old thrombi in 16 cases, and in some instances the connective tissue in organized thrombi showed the same basophilic change as that in the appendage.

Clinical Correlation

In none of the cases was there clinical evidence of rheumatic activity preoperatively, using such criteria as increased white blood cell count, increased blood sedimentation rate, and temperature elevation.

Sixty-nine per cent of the cases in the series were female, and Aschoff lesions were found in 50 per cent of these, and in 42 per cent of the males. The likelihood of finding granulomatous lesions, that is, a positive biopsy, was found to vary inversely with the patient age. Twenty-one patients aged 30 years and under were included, and, of these, 76.2 per cent had positive biopsies, whereas only 40.4 per cent of those over 30 had such lesions. The presence of auricular fibrillation preoperatively bore no relationship to the incidence of Aschoff lesions. Only 3 (14.3 per cent) of the patients less than 30 years of age were fibrillating, and, of these, one had a positive biopsy, while 29 (50.9 per cent) of those over 30 years were fibrillating, and only 4 of these had positive biopsies.

Thrombi, recent and/or old, were found in 33 cases (42.3 per cent) and 28 of these patients were fibrillating either preoperatively (22 cases) or postoperatively (6 cases). As noted, 32 had auricular fibrillation.

Table II

Postoperative Clinical Results Compared with Incidence of Positive Auricular Findings

Results	No. of cases	Per cent with positive biopsy
Excellent	37	46.0
Good	16	44.0
Fair	11	36.5
Unchanged	8	50.0
Died	6	66.0

Patients with granulomatous lesions appeared to do as well postoperatively as the remainder (Table II).

Postoperatively, 8 cases developed clinical evidence of rheumatic activity. In the majority of these patients the recrudescence occurred within 2 months of operation. Of these, 5 had positive biopsy findings. In one of the 3 re-

maining cases, no changes of any kind were noted in the auricular appendage. In a ninth case, questionable clinical signs of activity appeared, but, here too, the biopsy specimen was negative.

Discussion

The most frequent finding was the basophilic alteration in connective tissues. This change could be demonstrated well with toluidine blue, the altered tissue staining pink or violet.

The tissue basophilia is not specific, and may be seen in myxedema, in degenerating fibrocartilage, and in the vessels in hypertension. The metachromasia is considered to be due to the presence of certain sulfated mucopolysaccharides. Taylor[7] reported the accumulation of this metachromatic substance in areas of medial degeneration associated with aortic atherosclerosis, and stated that it was intimately and constantly associated with fragmentation of the elastic fibers. In about half the cases of rheumatic infection, basophilia of the media of arterioles is said to occur. In the present study, the basophilia was found associated with granulomas in 23 cases (29.5 per cent), but the change was seen in the absence of granulomas in 19 cases (24.4 per cent), and the question arises as to whether the basophilia may be related to the increased tension and stress on the auricular wall consequent to mitral stenosis, or to continued rheumatic activity.

The possibility that the basophilia of connective tissues in the surgical material might be attributable to the manipulations necessary for its removal has been entertained. That this is probably not so is suggested by the finding of similar changes in auricular appendages from known cases of rheumatic fever at necropsy.

Two possibilities are suggested by the fact that granulomas were encountered more frequently in the younger age group. First, the rheumatic process, although sub-clinical, may be continuously active. Alternatively, the presence of lesions could be explained by recrudescences becoming less frequent with advancing age. According to the clinical histories, rheumatic fever was present in some of these cases from 6 to 41 years before operation. Of the 8 cases developing clinical activity postoperatively, 5 had granulomatous lesions at biopsy. This is not significantly greater than the incidence of these lesions in the entire series.

The frequency with which pathologic changes were present in the lungs appears to be of some practical importance. Vascular changes and fibrotic changes in the alveolar walls, although variable in degree,

doubtless are permanent. It is difficult to conceive of their resolution even after marked alteration in pulmonary hemodynamics following valvoplasty.

Summary

Portions of 78 auricular appendages, obtained in the course of mitral valvoplasty, were examined microscopically. In only 10 of them were lesions completely absent. Histologic evidence of rheumatic activity in the form of Aschoff nodules was found in 50 per cent of the cases, sometimes many years after the initial attack and in the absence of signs of clinical activity. These lesions were almost twice as frequent in patients under 31 years of age than in the remainder of the series. The change observed most frequently was basophilic connective tissue degeneration, which was present in 54 per cent of cases. The other lesions encountered included cellular infiltration in endocardium and myocardium in 51 per cent, fibrosis in 40 per cent, and intra-auricular thrombi in 42 per cent. Thrombi were most frequently found in association with auricular fibrillation, but no correlation could be found between Aschoff lesions and arrhythmias. The presence of Aschoff lesions and postoperative course could not be correlated.

In an incidental study, about one half of 29 specimens of lung taken for biopsy were found to show changes indicative of chronic pulmonary hypertension.

Acknowledgment is made to Dr. R. W. Rutherford, Fellow in Cardiology, Toronto General Hospital, for the clinical data presented in this paper, and to Dr. W. Bigelow, Assistant Professor of Surgery, for permission to use the material from his case.

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[*Illustrations follow*]

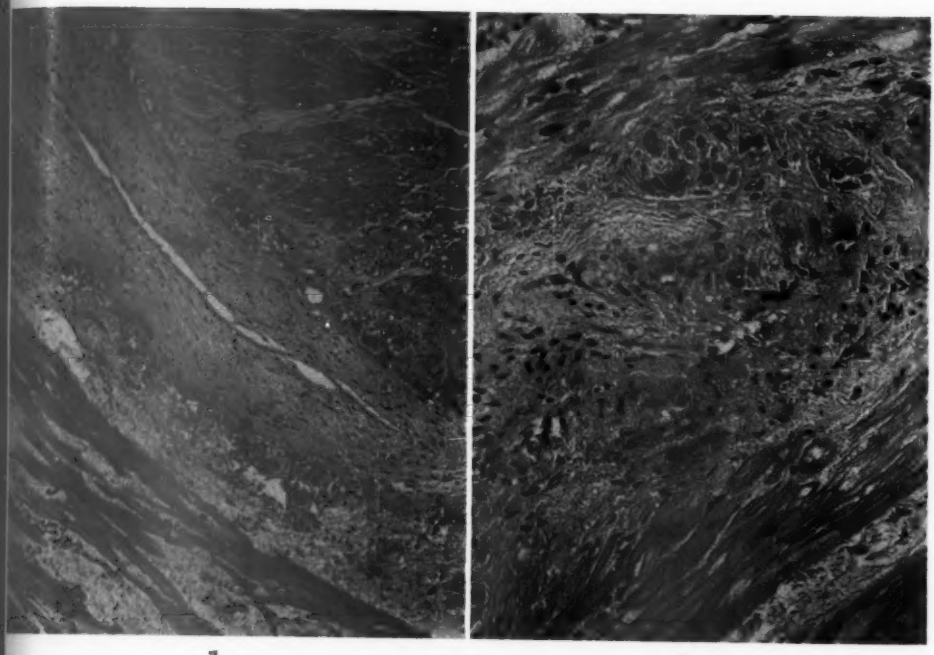
LEGENDS FOR FIGURES

FIG. 1. Subendocardial granulomas in a continuous band. Hematoxylin and eosin stain. × 81.

FIG. 2. Myocardial lesion consisting of a group of Aschoff nodules. Hematoxylin and eosin stain. × 154.

FIG. 3. Details of granulomatous lesions. Hematoxylin and eosin stain. × 308.

FIG. 4. Details of granulomatous lesions. Hematoxylin and eosin stain. × 308.



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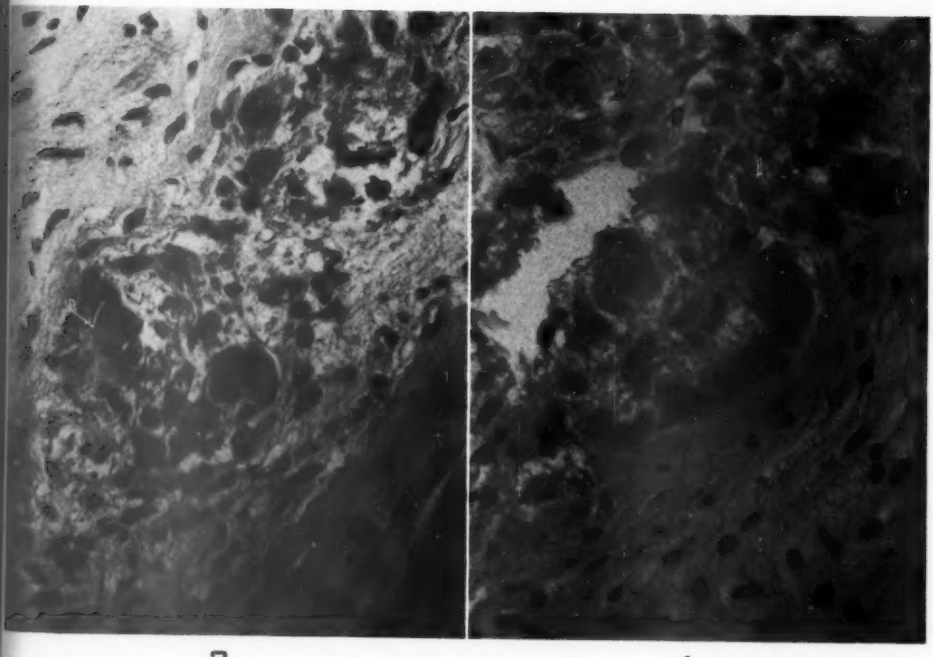

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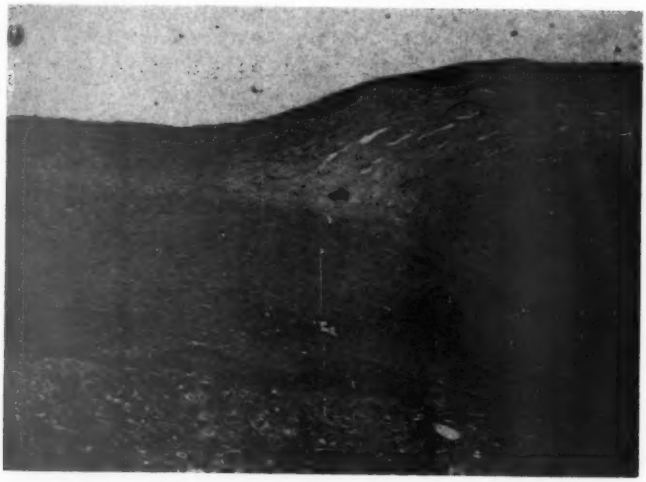
FIG. 5. Organized auricular mural thrombi. Hematoxylin and eosin stain. × 40.

FIG. 6. Separation of muscle fibers by swollen connective tissue. Hematoxylin and eosin stain. × 230.

FIG. 7. Organized auricular mural thrombi, with canalization. Hematoxylin and eosin stain. × 40.



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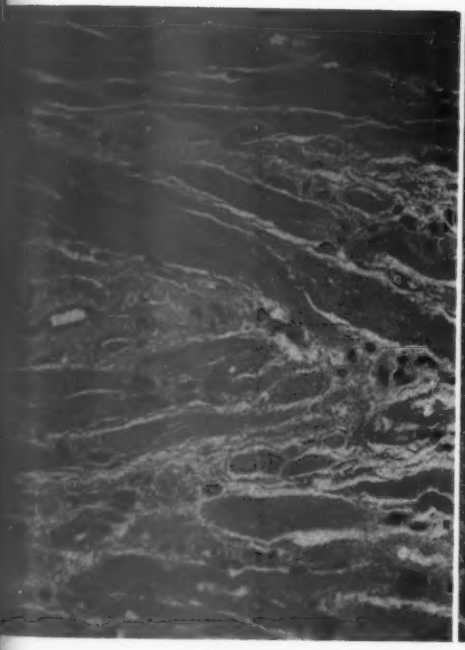

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7

BACTERIAL AORTITIS AND MYCOTIC ANEURYSM OF THE AORTA

A Report of Twelve Cases *

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Because of the resistance of the large elastic arteries to bacterial infection in general, mycotic (bacterial) aneurysms of the aorta are among the more uncommon encountered in that artery. Infection in the arterial wall is rarely due to fungi and the inappropriateness of the term mycotic is generally recognized, but it has the advantage of long and common usage since it was applied to these lesions by Osler.[1] It is the purpose of this communication to report 12 cases in which there was bacterial infection of the aortic wall with formation of an aneurysm in 9 of these cases. The 3 cases without aneurysm formation do not differ fundamentally in pathogenesis and are included for that reason. Three cases included in this study have previously been reported.[2-4]

Incidence

During the 50-year period of 1902 to 1951, 22,792 necropsies were performed at the Boston City Hospital. In these cases, 338 aortic aneurysms were encountered: 143 syphilitic, 92 arteriosclerotic, 78 dissecting, 9 mycotic, and 16 unclassified. The incidence of aortic aneurysm was 1.5 per cent.

During the review of these cases, some observations were made on the incidence of the more common types of aortic aneurysm which are worthy of inclusion. Of the 143 syphilitic aneurysms, 124 occurred in men and 19 in women. The syphilitic lesions were found in the thoracic aorta in all but 2 cases. In 37 cases death was due to internal rupture of the aneurysm; external rupture was not noted. Most of the syphilitic aneurysms (76 per cent) were encountered in the age group 41 to 70 years. Similarly, a majority of the arteriosclerotic aneurysms occurred in men, 60 of the total of 92. In contrast to the predominantly thoracic location of the syphilitic aneurysms, 73 of the arteriosclerotic group were found in the abdominal aorta and but 13 in the thoracic aorta. In 6 cases there were multiple arteriosclerotic aneurysms. Most of these aneurysms (88 per cent) occurred in the age group 61 to 90 years.

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TABLE I

Data on Twelve Cases of Bacterial Aortitis

Case	Sex	Age	Location	Description	Other aortic disease	Source of infection	Type	Other findings
1	M	30	Descending thoracic	1.5 cm. aneurysm		Tuberculous lymphadenitis	Secondary	Miliary tuberculosis
2	M	34	Sinus of Valsalva	1.2 cm. aneurysm		Aortic endocarditis	Secondary	Lobar pneumonia
3	M	44	Ascending thoracic	Longitudinal slit, rupture with hemopericardium	Atherosclerosis, cystic medionecrosis		Primary	Calcific aortic stenosis
4	F	29	Ascending thoracic	3 cm. aneurysm		Subacute bacterial endocarditis	Secondary	Trivalvular rheumatic heart disease
5	M	39	Sinus of Valsalva	3 cm. aneurysm, rupture and hemopericardium	Atherosclerosis	Subacute bacterial endocarditis	Secondary	Rheumatic heart disease
6	M	53	Aortic valve ring	1 cm. aneurysm	Atherosclerosis	Bacterial endocarditis	Secondary	
7[3]	M	35	Ascending thoracic	Multilocular aneurysm, 6 x 4 x 3 cm.	Congenital hypoplasia	Cellulitis of foot	Primary	Splenic and renal infarcts, focal embolic glomerulonephritis
8[3]	M	39	Ascending thoracic	Fusiform and saccular aneurysms, 4 x 2 x 2 cm.	Syphilitic aortitis	Gonococcal arthritis	Primary	
9[4]	M	75	Ascending thoracic	0.5 cm. slit, rupture with hemopericardium	Atherosclerosis	Bacterial endocarditis	Secondary	
10	M	9 mo.	Descending thoracic	Slit communicating with esophagus		Mediastinitis	Secondary	
11	M	55	Descending thoracic	5 cm. aneurysm, rupture into right pleural cavity	Atherosclerosis	Pneumonia (?)	Primary	
12	M	68	Descending thoracic	10 cm. aneurysm, rupture into posterior mediastinum	Cystic medionecrosis	Pneumonia	Primary	

Observations made on the frequency with which the various types of aortic aneurysms were encountered during the 50-year period indicate an increase in incidence of arteriosclerotic aneurysms, paralleling an increase in the average age of patients necropsied, and a sharp drop in the incidence of syphilitic aneurysms, attesting to the efficacy of improved methods for the treatment of syphilis. In the first 4 decades, 1902–1941, syphilis was responsible for 52 to 70 per cent of all aortic aneurysms. In the last decade, however, there was a drop to 25 per cent. Contrariwise, there was noted a steady rise in the incidence of arteriosclerotic aneurysms until they accounted for 43 per cent of all aortic aneurysms in the last decade included in the study, 1942–1951. These observations are in agreement with those of Maniglia and Gregory.[5] Dissecting aneurysms were not studied in detail.

The 12 cases of bacterial aortitis and mycotic aneurysm of the aorta showed neither a tendency to occur in a particular age group nor a detectable change in incidence over the 50-year period; but the number of cases is very small in relation to the total. Eleven of the 12 cases occurred in men; all of the lesions were found in the thoracic aorta. Pertinent pathologic data are collected in Table I.

Pathologic Findings

In size, the lesions ranged from a 1 mm. erosion to a 10 cm. sac (Figs. 1, 2, and 3). In 3 cases (nos. 3, 9, and 10), infection in the aortic wall had produced an erosion and rupture without aneurysmal dilatation. In 6 cases there was rupture of the lesion as follows: into the pericardial cavity in 3 cases, and into the right pleural cavity, the posterior mediastinum, and the esophagus in one case each. Microscopically, there was noted destruction of the intima and musculoelastic lamellae by an acute inflammatory process (Fig. 4). Heavy infiltrates of neutrophils and abscess formation were noted frequently (Figs. 5 and 6). Bacteria often were demonstrable in tissue sections. In older lesions and at the margins of acute inflammatory zones there often was active fibroblastic proliferation and formation of granulation tissue. It is of particular interest that, in those cases in which aneurysms were unassociated with bacterial endocarditis or infection in adjacent structures and in several other cases as well, study of the aorta showed the presence of additional aortic disease which included atherosclerosis, cystic medionecrosis (Fig. 7), syphilitic aortitis, and congenital hypoplasia.

An intravascular source for infection was apparent in 5 cases. In 2 cases (nos. 1 and 10) the source of infection was found in adjacent structures in the mediastinum. Of the 5 remaining cases there was a

putative source for infection in remote structures in 4: a history of cellulitis of the right foot in case 7, a history strongly suggestive of gonococcal arthritis in case 8, a history suggestive of pneumonia 6 weeks before in case 11, and organizing bronchopneumonia found at necropsy in case 12. In case 3, no source for the infection was apparent. The results of bacteriologic studies are indicated in Table II.

TABLE II

Infecting Organisms in Cases of Bacterial Aortitis

Case	Organism	How demonstrated
1	*Mycobacterium tuberculosis*	Direct smear
2	Pneumococcus, type 1	Ante-mortem blood culture
3		
4	Gram-positive cocci	Sections of aneurysm
5	*Streptococcus viridans* Gram-positive cocci	Culture of vegetations Sections of aneurysm
6	Pneumococcus, type 28 Beta hemolytic streptococci	Culture of vegetations Culture of vegetations
7	Gram-positive cocci	Sections of aneurysm
8	Pneumococcus, type 17 *Neisseria gonorrhoeae*	Post-mortem culture of blood, aneurysm, and spleen Post-mortem culture of aneurysm and spleen
9	Atypical coliform organism	Culture of vegetations, tissue sections
10	Pneumococcus, type 33 Fusospirochetal organisms Gram-positive cocci	Post-mortem culture of lungs Tissue sections Tissue sections
11	Pneumococci, diplococci (Gram-positive)	Ante-mortem throat culture Tissue sections
12	Diplococci (Gram-positive)	Tissue sections

The bacteriologic findings indicate the prominence of Gram-positive cocci, particularly the pneumococcus, as causative organisms in these cases. These cocci, when identified culturally, were found to be pneumococci in 5 cases and streptococci in one case. Both gonococci and pneumococci were isolated in case 8. *Mycobacterium tuberculosis* and an atypical coliform organism were found in one case each (nos. 1 and 9). There was one case of fusospirochetal infection (case 10).

ILLUSTRATIVE CASES

Case 2

A 34-year-old white man was admitted with a history suggestive of lobar pneumonia and physical findings were compatible with that diagnosis. Blood cultures

were positive for a pneumococcus, type 1. On the 39th hospital day, the patient developed high fever with rising pulse and respiratory rates. He suddenly developed extreme dyspnea and died rapidly on the 41st hospital day.

At necropsy there was lobar pneumonia of the right lung. On the left anterior aortic valve cusp, near the edge, there was a vegetation 7 mm. in diameter. Above the right anterior cusp was an aneurysm of the sinus of Valsalva, 1.2 cm. in diameter. On microscopic examination of sections taken through the involved aortic cusp and adjacent aneurysm there was necrosis and destruction of the cusp with a typical bacterial vegetation. The aneurysm contained laminated blood clot and the underlying aortic wall was heavily infiltrated with neutrophils and contained scattered abscesses (Fig. 6). Masses of cocci were present in the sections.

Case 3

A white man, 44 years old, who did not appear acutely ill, was admitted to the hospital because of lassitude, fatigue, and malaise. The heart was enlarged to the left and a rough systolic murmur at the apex entirely replaced the first sound. There was also a short, blowing, mid-diastolic murmur at the apex. Over the second, third, and fourth left intercostal spaces was a loud friction rub. The clinical diagnosis was pericarditis. The patient died quietly in his sleep.

At necropsy there was an acute fibrinous pericarditis and hemopericardium (200 cc.). The aortic valve was thickened, with adherence of two cusps, presenting the appearance of nodular calcific aortic stenosis, but without evidence of fresh endocarditis. There was an irregular, longitudinal slit, 1.5 cm. above the aortic valve. The borders of this slit were raised, irregular, and bore small vegetations. A probe passed through it entered the pericardial cavity. On microscopic examination of the aorta, there was atherosclerosis and cystic medionecrosis (Fig. 7) with dissection. In the region of the perforation there was destruction of the aortic wall by an acute inflammatory process, with deposits of fibrin and neutrophils, formation of abscesses, and marginal fibroblastic proliferation.

Case 10

A white male infant, 9 months of age, was admitted because of anorexia, restlessness, and fever for 1 week. On the day before admission there were two episodes of hematemesis. Fifteen minutes after admission there was another bout of hematemesis and the patient died. There was no history of ingestion of a foreign body.

At necropsy, there was found just beyond the origin of the left subclavian artery a small area, 0.5 cm. in diameter, in which the aorta appeared thinned and slightly discolored. In the center of this area was a small hole, 1 mm. in diameter (Fig. 1), through which a probe could be passed into the esophagus. Between the aorta and the esopha-

gus was a fistulous tract lined by ragged, soft red tissue. In the esophagus was a longitudinal slit, 8 mm. in diameter, which communicated with the sinus tract. The gastrointestinal tract contained large amounts of dark red blood. On microscopic examination the fistulous tract was found to be lined with necrotic débris and fresh granulation tissue. There were many collections of neutrophils and small abscesses. Bacterial stains revealed a variety of organisms, including short fusiform bacilli, large spirochetes, cocci in short chains and clusters, and short bacilli.

Case 11

A 55-year-old man was admitted to the hospital because of right anterior chest pain. He had had chronic cough with expectoration for many years, with occasional attacks of pain in the left knee and ankle. Two years previously he had had several abscesses drained in the right foot and had not worked since that time. Six weeks before admission he had a bout of stabbing right upper abdominal pain aggravated by inspiration and coughing. Temperature was 100.8° F. On physical examination there was dullness over the entire right hemithorax with diminished breath sounds over that area. Serologic test for syphilis was negative. Culture of the sputum yielded a pneumococcus. Roentgenograms of the chest showed an increase of bronchovascular markings on the right and slightly diminished radiolucency in the right middle and lower lung fields. These changes were interpreted as suggestive of pneumonitis. The patient was treated with penicillin. Ten hours after admission he had a generalized convulsion and expired within 40 minutes.

At necropsy the right pleural cavity contained a large blood clot weighing 3,000 gm. Just above the crura of the diaphragm there was an aortic aneurysm, 5 cm. in diameter. In the intimal surface this was apparent as a shallow depression with ragged edges and a slit-like tear in the center (Fig. 2). On microscopic examination all coats of the aorta were infiltrated with neutrophils, often aggregated into small abscesses (Fig. 5). There was partial necrosis and fragmentation of the medial lamellae, particularly in the region of the perforation which communicated with the right pleural space. Paired Gram-positive cocci were identified in appropriately stained sections of the aortic wall.

Case 12

A white man, 68 years old, was admitted because of severe substernal and epigastric pain of 6 hours' duration. During the 7 years before admission, he had had infrequent paroxysms of dyspnea, wheezing, and cough which were not incapacitating. Six months before admission he developed increasing hoarseness, for which a cause was not found on laryngoscopic examination. About 6 hours before admission he complained of excruciating substernal pain associated with severe dyspnea, cyanosis, and sweating. Physical examination was negative except for rhonchi and wheezes in both lungs. The temperature was 101° F.; pulse rate, 120; respirations, 30; leukocyte count, 18,000 per cmm. with 81 per cent neutrophils. Serologic test for syphilis was negative. Slight deviation of the trachea and superior mediastinal contents to the right was noted on roentgenologic examination of the chest. A barium study of the

esophagus showed almost complete extrinsic obstruction in the middle third. Two episodes similar to the admitting complaint occurred during the following 9 days. Shortly after the second such episode, the patient died.

At necropsy, there was a fusiform aneurysm of the descending thoracic aorta, 10 cm. in length and 13 cm. in maximum circumference. It had ruptured anteromedially in the plane between the aorta and esophagus, and had compressed the esophagus in its middle third (Fig. 3). The presence of old blood clot and fibrous tissue deposition in this area suggested previous leakage. The recurrent laryngeal nerve was not identified. In microscopic sections there was destruction of all layers of the aortic wall with fragmentation of elastic fibers (Fig. 4). On the inner surface of the aneurysm there were layers of old laminated blood clot. A neutrophilic and monocytic infiltrate extended through all coats of the aorta and there were abscesses in the adventitia. Small numbers of paired Gram-positive cocci were seen in appropriately stained sections.

Discussion

Bacterial infections of the aortic wall may be of intravascular or extravascular origin. In those of intravascular origin the source of infection is more commonly the vegetations of bacterial endocarditis, as in cases 2, 4, 5, 6, and 9 of the present series. These lesions commonly are found in the sinuses of Valsalva as a result of the extension of aortic endocarditis to involve the proximal portion of the aorta. Some infections of the aortic wall are of extravascular origin and arise by extension from adjacent lesions such as masses of tuberculous lymph nodes or abscesses. Cases 1 and 10 are illustrative of this mode of pathogenesis, the aortic infection having extended from an adjacent focus of tuberculous lymphadenitis in the former case and from mediastinitis, presumably the result of perforation of the esophagus, in the latter.

Bacterial infections of the aortic wall also occur in cases in which there is neither a demonstrable intravascular nidus of infection, as bacterial endocarditis, nor infection in adjacent structures. In such cases infection may originate in distant foci and be disseminated by the blood stream. Aneurysms in which this pathogenesis is postulated have been termed primary mycotic aneurysms by Crane,[2] who defines this entity as "a lesion developing in the wall of an artery which is not associated with any demonstrable intravascular inflammatory focus, as bacterial endocarditis, or with any inflammatory process in the surrounding tissues." To apply the term primary to aneurysms of this

sort is not to deny them causality, since reasonable sources for blood stream infection can be found in most cases. Of the five primary mycotic aneurysms in the present series, plausible sources for infection were available in four. In case 7, the patient had recently been treated for cellulitis of the foot. In case 8, in which the aneurysm was of gonococcal origin, the history was strongly suggestive that the patient had had gonococcal arthritis. The history of case 11 suggested pneumonia 6 weeks before admission to the hospital and, in case 12, an organizing bronchopneumonia was found at necropsy. Of the 24 cases of primary mycotic aneurysm reported prior to 1945,[6] most of the primary infections were in soft tissue, bone and joints, and in the lung.

Any artery may be involved by bacterial infection in the ways described but the aorta is the more frequently affected,[7] favorite sites being the root and ascending thoracic portions. Next most commonly involved are the abdominal arteries, particularly the superior mesenteric, hepatic, and splenic. In these vessels infection is one of the more common causes of aneurysm. The intracranial arteries, particularly the middle cerebral, and the large vessels of the extremities are the next most frequently involved.

The means by which bacteria gain access to the aortic wall in cases in which there is direct extension of the inflammatory process from an infected aortic valve or from diseased mediastinal structures is obvious. The means by which organisms gain access in cases in which there is no continuity between the involved structures have been discussed by Stengel and Wolferth[7] and by Rappaport[8] and may be summarized as follows: first, that bacteria or infected emboli settle or lodge on the intimal surface of the artery; second, that bacteria or infected emboli lodge in the vasa vasorum. Septic emboli may bring infected material directly in contact with vessel walls in such situations as at the bifurcations of the cerebral arteries, but direct infection of the intact, undiseased intima of a large elastic artery, such as the aorta, must be extremely rare or non-existent.

That bacteria may gain access to the aortic wall by way of the vasa vasorum is illustrated by two reported cases. Owens and Bass[9] reported a case of tuberculous aneurysm of the abdominal aorta in which there was no adjacent tuberculous focus. It was their opinion that the organisms entered the aorta via the vasa vasorum. In a case of bacterial aortitis complicating syphilitic aortitis, reported by Rappaport,[8] there were multiple miliary abscesses in the media and intima and it is likely that, in this case also, the infection was transmitted by the vasa vasorum.

It is significant that, in the present series, except in those cases in which there was a demonstrable intravascular or adjacent focus of infection, underlying disease was found in the aorta in all cases. Congenital hypoplasia of the aorta was noted in case 7. In discussing the high incidence of bacterial infection and mycotic aneurysm in the adult type of coarctation of the aorta, Abbott[10] has pointed out that the dilated and atheromatous aorta below the stenosis, and the kinks and deformities of the constriction provide a favorable nidus for the lodgment of bacteria which might otherwise be quite avirulent. Thus the vicinity of a coarctation, like other congenital defects, provides a definite *locus minoris resistentiae.*

Rappaport[8] also has mentioned that the ulcerated, atherosclerotic aortic intima may provide a suitable site for bacterial infection. Syphilitic aortitis was a complicating factor in case 8 of this series. It is of interest to note that, in an early case of mycotic aneurysm described by Osler,[1] the lesion was engrafted on a syphilitic aorta. In a case of mycotic aneurysm of the abdominal aorta with dissection, reported by Lippincott,[11] syphilitic aortitis was believed to have been present also. It is reasonable to suppose that the wrinkled plaques and increased medial vascularization of the syphilitic aorta may increase the hazard of bacterial infection. A case of suppurative aortitis with cystic medionecrosis, dissection, and perforation of the aorta has been reported by Williams,[12] and it is possible that medial degeneration may somehow facilitate the establishment of infection in the aortic wall. Medial dissection has been recorded occasionally in cases of bacterial aortitis or mycotic aneurysm. Dissection was noted in cases 3 and 9 of the present series and also in Lippincott's case. In neither of the last 2 cases was medial degeneration or cystic necrosis noted, but it was present in case 3 of the present series.

Summary

Twelve cases in which there was bacterial infection of the aortic wall have been presented. In 9 cases there was formation of a mycotic aneurysm. In 7 cases the source of infection was bacterial endocarditis or disease in adjacent mediastinal structures. In 5 cases, the source of infection was remote (primary mycotic aneurysms). These aneurysms represent an incidence of 2.6 per cent of 338 aneurysms occurring in 23,000 necropsies. All of the aneurysms were thoracic in location. In no case had the diagnosis been made before death.

Underlying aortic disease was found in many cases. These processes included atherosclerosis, cystic medionecrosis, congenital hypoplasia,

and syphilitic aortitis. This observation suggests that pre-existing disease of the aorta favors the establishment of infection in it.

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LEGENDS FOR FIGURES

FIG. 1. Case 10. Slit lesion in aorta below origin of left subclavian artery, communicating with esophagus.

FIG. 2. Case 11. Primary mycotic aneurysm of descending thoracic aorta. Rupture into right pleural cavity.

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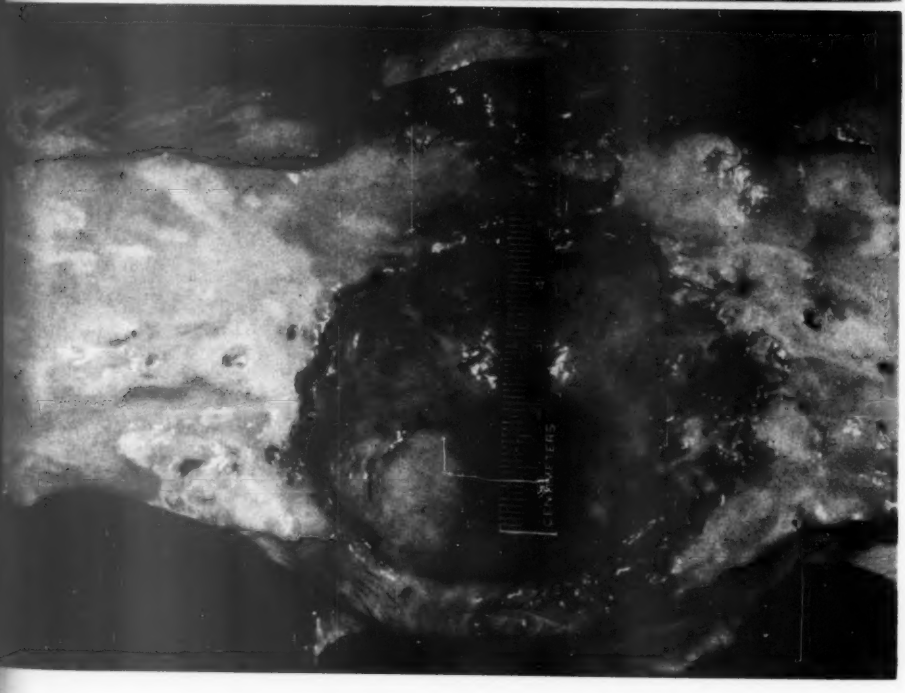

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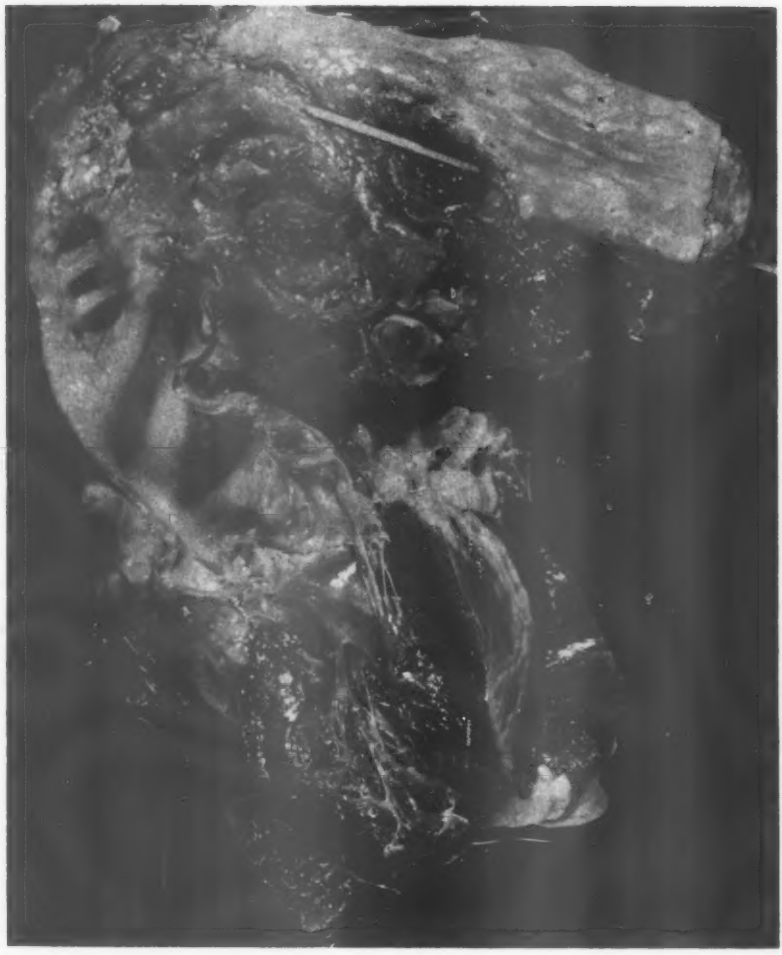

FIG. 3. Case 12. Primary mycotic aneurysm of descending thoracic aorta. Rupture into posterior mediastinum with compression of esophagus.

FIG. 4. Case 12. Edge of aneurysm, showing destruction of elastic fibers. Verhoeff-van Gieson's stain. × 46.

FIG. 5. Case 11. Neutrophilic infiltration with abscess formation in intima. Phloxine-methylene blue stain. × 32.

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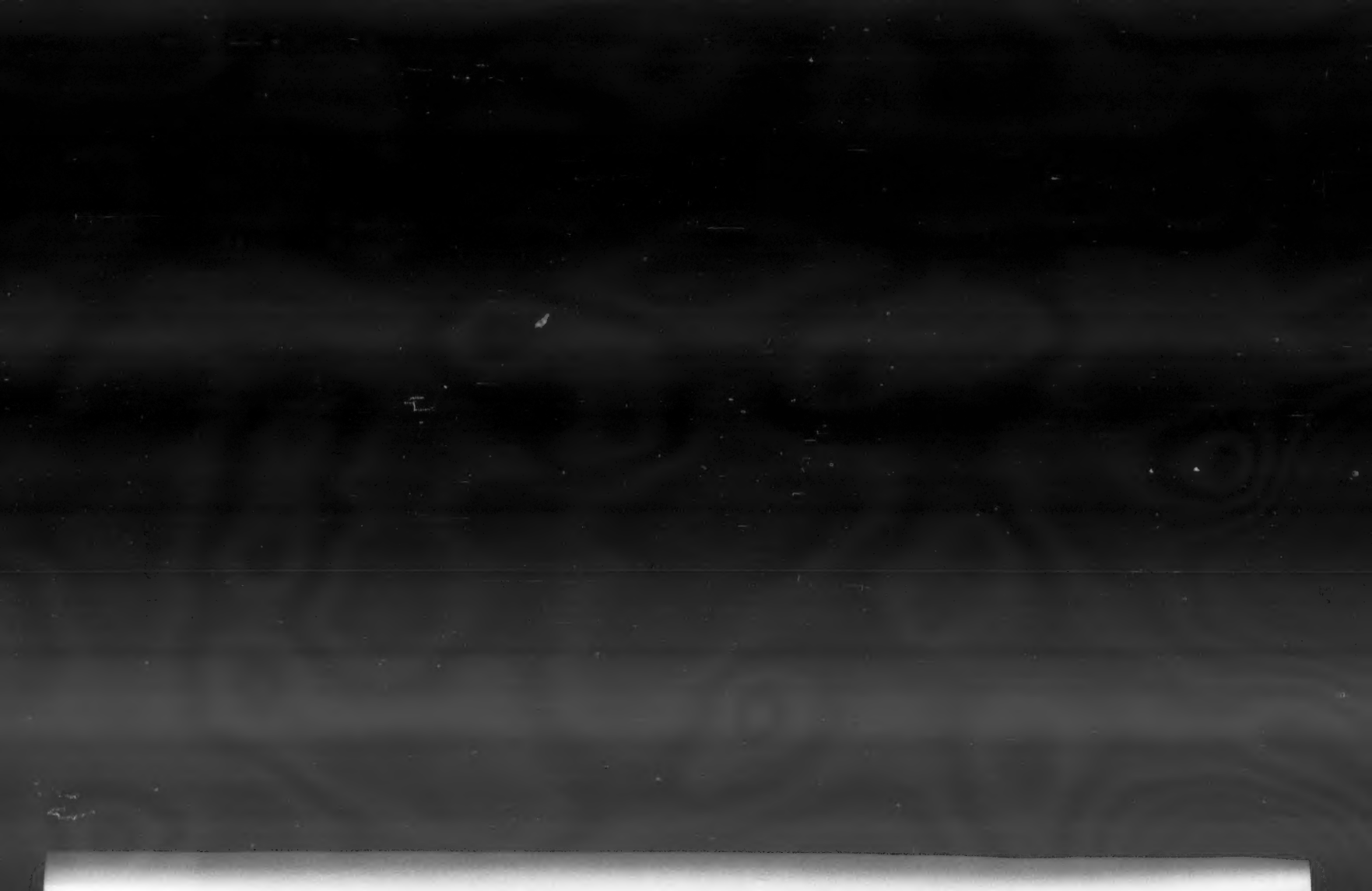
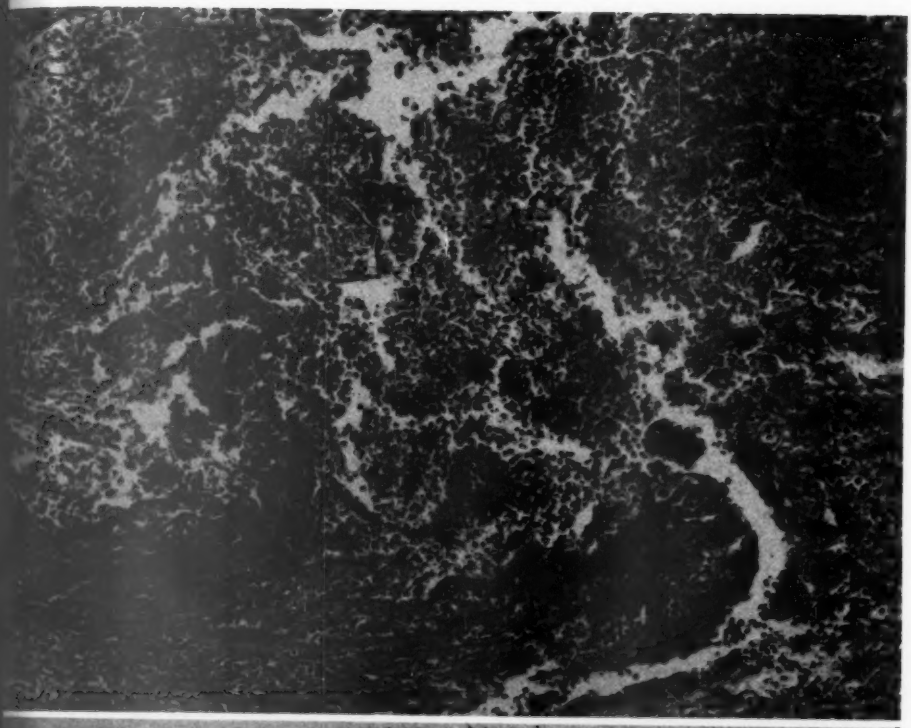
FIG. 6. Case 2. High-powered view of medial abscess, showing fragmentation of elastic fibers. Phloxine-methylene blue stain. × 740.

FIG. 7. Case 3. Portion of uninvolved aorta, showing areas of cystic medionecrosis. Phloxine-methylene blue stain. × 74.





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PERIARTERITIS NODOSA LIMITED TO THE PULMONARY CIRCULATION *

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Although periarteritis nodosa is a relatively uncommon disease, it has aroused considerable interest and controversy since its original description by Kussmaul and Maier.[1] Medical literature contains numerous reports, both clinical and pathologic, of pertinent vascular lesions in every conceivable anatomical location. Many of these indicate the pulmonary circulation as a site of localization, but in relatively few are the lungs the sole organ involved. In still fewer are the significant pathologic findings in the lungs satisfactorily documented.

In view of the extensive current investigation into the pathogenesis of this entity, it is believed appropriate to report the following 6 cases of periarteritis nodosa, limited to the lungs and associated, in each case, with pulmonary hypertension.

REPORT OF CASES

Case 1

N. H. was a white female, 28 years old, who was admitted to Cincinnati General Hospital (no. 209144) on December 17, 1945, with chief complaints of orthopnea and dyspnea. At age 21, she had had acute rheumatic fever, and 2 years prior to admission she had been told that she had a cardiac valvular lesion. Since that time she had suffered frequent attacks of dyspnea. Two weeks before her final hospital admission, she had a sudden onset of persistent shortness of breath and weakness. Three days prior to admission, edema, abdominal swelling, and hemoptysis had appeared.

On admission, the pulse was 80; respirations, 36; blood pressure, 140/70 mm. of Hg; temperature, 100.4° F. The patient manifested acute respiratory distress and cyanosis. Examination of the chest revealed numerous rhonchi and moist râles and wheezes in both lungs. There was dullness to percussion at the left lung base. Cardiac findings included a coarse aortic systolic murmur and mitral systolic and diastolic murmurs, a regular sinus rhythm with an apical cardiac rate of 80, and cardiomegaly. Marked peripheral edema also was noted. Laboratory studies revealed slight anemia and an initial normal white blood cell and differential count, rising ultimately to 10,700 white blood cells per cmm. The urine showed 3 plus albumin and five white blood cells and ten erythrocytes per high-power field. Four blood cultures were negative. Electrocardiograms on two occasions showed a digitalis effect and evidence of acute myocardial damage. Roentgenographic and fluoroscopic examinations of the chest revealed cardiomegaly with a mitral configuration, and perivascular pulmonary infiltrates.

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The patient was treated with digitalis, aminophyllin, and sedation. Antibiotics were not administered despite intermittent febrile episodes. The course was characterized by increasing respiratory distress terminating in a fatal bout of dyspnea on December 18, 1945.

Necropsy (N-45-505) was performed 5 hours after death. Externally, the body exhibited peripheral edema. There were bilateral serous pleural effusions. The heart weighed 475 gm. and showed hypertrophy and dilatation of all chambers. The left ventricle was least affected, measuring 17 mm. in thickness, while the right ventricular wall measured 7 mm. in thickness. There was a tight mitral stenosis with thickening, fusion, and calcification of the cusps. The aortic valve showed moderate deformity and calcification with stenosis. The lungs were enlarged, the right weighing 1,110 gm. and the left, 860 gm. The grayish red, right upper lobe appeared consolidated. The other lobes were subcrepitant and edematous. The liver and spleen were enlarged and congested. The granular kidneys had a combined weight of 495 gm. The head was not examined, and other gross findings were not contributory.

Microscopic examination of the heart showed scarring and vascularization of both the mitral and aortic valves and their rings. No Aschoff nodules were encountered. The lungs exhibited the typical changes of chronic passive congestion,[2] and severe sclerosis of arterioles and smaller pulmonary arteries. Many of the latter revealed fibrinoid necrosis of the walls with scarring and massive mural and perivascular inflammatory infiltrates. The reacting cells were largely neutrophils (Fig. 1). The walls of some vessels were completely destroyed, but thrombosis was not evident. Sections of the kidneys showed acute glomerulonephritis, but no arteritis. Sections of bladder, aorta, liver, pancreas, spleen, stomach, small intestine, colon, adrenal glands, uterus, and ovary failed to reveal any evidence of angiitis.

Case 2

C. N., a 16-year-old white girl, was admitted to Bethesda Hospital (no. 186309) on August 29, 1947, with a complaint of headache of 2 days' duration. Two and one-half years prior to admission, following a bout of fever, a physician had told her that she had rheumatic heart disease. She had remained asymptomatic until the recent episode, which terminated in convulsive seizures on the day of admission.

On physical examination, the temperature was 101° F.; pulse, 120; respirations, 30; and blood pressure, 112/76 mm. of Hg. The patient was semicomatose, irritable, and incontinent. Slight nuchal rigidity was noted. The lungs were clear to percussion and auscultation, and a grade IV systolic murmur was heard at the cardiac apex. Neurologic examination revealed no specific changes. Laboratory examinations showed an admission white blood cell count of 24,000 per cmm. with a normal differential count. The hemoglobin was 8.8 gm. per 100 cc. with a proportionate erythro-

cyte count. Subsequently, the hemoglobin did not vary, but the white blood cell count fell to 9,000 per cmm. in 10 days, only to rise thereafter to a level of 30,000 per cmm. with a neutrophilic leukocytosis on the day of death. Erythrocyte sedimentation rate was consistently above 30 mm. per hour. Cerebrospinal fluid examination during the first week of hospitalization revealed a white blood cell count varying from 9 to 108 cells per cmm. These were largely lymphocytes, and the protein of the spinal fluid ranged between 76 and 132 mg. per 100 cc.; but three cultures of the spinal fluid were negative. On October 10, blood culture was negative. Roentgenographic examination of the chest on admission revealed pulmonary congestion, pulmonary infiltrates, and cardiomegaly. Three weeks later, there were pleural effusions, and subsequent films showed accentuation of the previously described changes. Electrocardiograms demonstrated tachycardia, prolonged conduction time, and right axis deviation.

The patient received sedation because of bizarre, varying, neurologic findings and periodic disorientation. She remained febrile, with her temperature in the first week reaching levels as high as 106° F. Two days after admission, digitalis and oxygen therapy was initiated because of the presence of râles, cyanosis, and a feeble pulse. Neurologic and respiratory signs diminished, and administration of salicylates was begun on the following day. By September 15, she was much improved and required no oxygen therapy. She remained in this condition until October 6, when she suffered a paroxysm of nocturnal dyspnea, and hepatomegaly was discovered. She failed to respond to oxygen therapy and developed oliguria 2 days later. During the following 2 weeks, dyspnea increased, oliguria persisted despite attempts at diuresis, and cyanosis and anasarca appeared. On October 24, 1947, her symptoms worsened, her fever rose to 103.6° F., and she expired.

Necropsy (A-908) was performed shortly after death. Externally, there were noted edema, cyanosis, and malar flush. Both pleural cavities contained serous effusions. The heart weighed 375 gm. and manifested right ventricular and atrial enlargement, with dilatation of the pulmonic and tricuspid rings. The right ventricular wall measured 7 mm. in thickness, while the left ventricular wall was of normal thickness. The only valve showing deformity was the mitral valve, which was tightly stenotic. The lungs had a combined weight of 1,582 gm. and showed diminished crepitation. There was increased resistance to sectioning, and the cut surface appeared pinkish white. A loosely adherent thrombus was noted in a single secondary branch of a pulmonary artery. The only other pertinent findings in this complete necropsy were ascites and visceral congestion. The brain appeared normal grossly.

Microscopically, the myocardium revealed scattered Aschoff nodules. In the lungs, evidence of severe chronic passive congestion and sclerosis of pulmonary arteries and arterioles were prominent findings. Scattered interstitial fibrin precipitates with neutrophilic reaction were seen. An organizing arterial thrombus supplying an area of healing infarction was present. Many small and medium-sized arteries showed mural fibrinoid necrosis with neutrophilic inflammatory exudates with-

in their walls and in surrounding tissues (Fig. 2). Others exhibited excentric mural scarring and scant histiocytic exudate with narrowing of their lumina. In a large vessel, there were intimal edema, fibrinoid necrosis, and histiocytic accumulations producing a narrowing of the lumen (Fig. 3). Sections of the liver, pancreas, spleen, kidneys, stomach, and brain revealed only congestion, but no evidence of angiitis.

Case 3

P. M. M. was a white boy, 7 years of age, who was admitted to the University Hospital, Ann Arbor, Michigan (no. 718752), on December 23, 1951, with complaints of recurrent attacks of exertional dyspnea and of syncope of 1 month's duration. Five days prior to admission, he had suffered a bout of hacking cough, vomiting, lethargy, and severe shortness of breath. He was intermittently febrile and was given antibiotic therapy at another hospital before entry. There, an electrocardiogram revealed an increased conduction time and right axis deviation.

On admission, physical examination revealed a well developed boy whose temperature was 99.2° F. There were hyperpnea and a paroxysmal, non-productive cough. Scattered, dry rhonchi were heard throughout both lung fields. The heart was slightly enlarged, but no murmurs were heard. There was a loud pulmonic second sound. The remainder of the physical examination was not remarkable. Laboratory findings included a normal hemoglobin content and a white blood cell count of 3,600 per cmm. with 92 per cent neutrophils. Platelet count and erythrocyte sedimentation rate were normal. Urinary findings were not remarkable. Cultures of the blood and sputum showed no pathogenic organisms. Roentgenographic and fluoroscopic examination of the chest revealed cardiomegaly, accentuation of the right border of the heart, and prominent pulmonary vessels. An electrocardiogram showed evidence of right ventricular hypertrophy.

The patient was treated with bed rest, low sodium diet, phenobarbital, salicylates, and antibiotic therapy. The temperature remained normal, and the pulse dropped to normal levels. At the time of discharge, January 4, 1952, he manifested no dyspnea while resting. He was scheduled to return on January 30 for cardiac catheterization studies, but, on January 14, he had an episode of lethargy and cyanosis and was brought to the clinic, where dyspnea and cough were noted. A blowing systolic murmur over the precordium varied with the respiratory cycle. White blood cell count was 10,200 per cmm. with a normal differential count. He was sent home and, on January 17, 1952, died under unknown circumstances.

Necropsy (A-255-BD) was performed 12½ hours post mortem. Pertinent gross findings were limited to the chest, all other organs examined in this complete necropsy revealing only evidences of passive congestion. The heart was enlarged, weighing 200 gm. Hypertrophy and dilatation were limited to the right chambers. The left ventricular wall measured 6 mm. in thickness, while the right ventricular wall measured 10 mm. There was dilatation of the pulmonic ring and the pulmonic arteries. The left lung weighed 170 gm.; the right lung, 190 gm. There was diminished crepitation with evidence of congestion. The cut surface appeared mottled yellowish pink. Bronchial lymph nodes revealed congestion and prominence of follicles.

Microscopic examination of the right ventricular myocardium showed hypertrophy of muscle fibers only. No inflammatory lesions were noted. The lungs exhibited marked sclerosis of most pulmonary arteries and arterioles, many containing hyaline thrombi. Medium-sized and small arteries revealed patchy, mural, fibrinoid necrosis and neutrophilic infiltrates with occasional lymphocytes and histiocytes (Fig. 4). Many arteries contained thrombi with canalization, and others had mural defects replaced by scar. The lungs, in general, showed congestion, edema, and histiocytic collections in alveolar septa. Other organs examined microscopically included spinal cord, brain, thyroid gland, esophagus, thymus, spleen, small intestine, colon, stomach, pancreas, liver, adrenal glands, kidneys, skin, muscle, testis, and bladder. The only finding of significance was passive congestion. No arteritic lesions were detected in these organs.

Case 4

A 47-year-old white female, M. H., was admitted to Cincinnati General Hospital (no. 286670) for mitral valvulotomy on June 16, 1952. Her illness had begun in adolescence, when she had suffered from "growing pains" and frequent sore throats. She had no further symptoms until 14 to 15 years prior to admission, when she sought medical care for shortness of breath. Cardiomegaly was discovered, and she was treated with a digitalis preparation, which she continued to take until the time of her admission. She remained symptom-free until 4 years prior to admission when her dyspnea became aggravated, and evidence of right-sided cardiac failure appeared. From that time on, symptoms of dyspnea, cyanosis, and orthopnea gradually increased, until, on admission, she was bedridden.

Physical examination revealed a temperature of 98° F. The pulse was irregular, 116; respirations, 24; blood pressure, 120/80 mm. of Hg. The patient appeared chronically ill and exhibited malar flush and venous distention. Wheezes were heard in both lung fields. The apical cardiac rate was 112 and grossly irregular. A booming mitral first sound and apical systolic and diastolic murmurs were heard. The tender edge of the liver was palpated 2 fingerbreadths below the right costal margin. Moderate pretibial pitting edema was the only other significant finding. Laboratory findings included a normal hemoglobin value and a white blood cell count of 12,000 per cmm. This rose terminally to 25,500 per cmm. with 94 per cent neutrophils. Aside from 1 plus albuminuria, the urine was normal. Blood chemical constituents were not remarkable except that the sodium was 114 to 118 meq. per l. Two blood cultures were negative. An electrocardiogram showed auricular fibrillation, digitalis effect, and evidence of right ventricular hypertrophy. On June 18, fluoroscopic and roentgenographic examination of the chest demonstrated enlargement of the right side of the heart, enlarged pulmonary arteries, and prominence of the right hilum. On June 24, a shadow appeared in the right costophrenic angle and obliterated the right border of the heart. By July 4, parenchymal infiltrates and pleural effusions were noted.

The patient was treated with mercurial diuretics, a low sodium diet, and digitalis. On June 20, cardiac catheterization was performed, revealing a resting arterial oxygen saturation of 80 per cent. Dyspnea gradually worsened, and, on July 2, râles were detected in the lungs. Because of leukocytosis and despite the absence of fever,

she was given penicillin and oxygen therapy with transient improvement on the following day. However, the symptoms recurred, and the course continued downhill, resulting in death on July 7, 1952.

Necropsy (N-52-349), restricted to examination of the thoracic viscera, was carried out 1½ hours post mortem. Externally, scleral icterus and peripheral edema were noted. Both pleural cavities contained serous effusions. The right lung weighed 920 gm.; the left, 575 gm. The former showed diminished crepitation and firmness of consistency in its lower two thirds. The cut surface was mottled yellowish red, and the lower lobe appeared to contain little air. The bronchi were congested. The right pulmonary artery was dilated and contained a huge, purplish red, friable, somewhat adherent thrombus, completely filling its lumen. The main pulmonary artery was markedly dilated. The cut surface of the left lower lobe resembled that of the right, but with less loss of crepitation. There was moderate cardiomegaly, the heart weighing 315 gm. The enlargement was largely right-sided, with dilatation of both the pulmonic and the tricuspid rings and hypertrophy of the right ventricular wall to a thickness of 7 mm., while the left side had a thickness of 11 mm. Severe stenosis and calcification were present in the mitral valve, and there were left atrial hypertrophy and dilatation. The aortic valve showed slight scarring with thickening and calcification. The other thoracic viscera were not remarkable.

Microscopic examination of the heart revealed focal perivascular scarring and myocardial hypertrophy. The mitral valve showed hyaline fibrous thickening, and the aortic valve manifested a similar process to a less pronounced degree. There were no Aschoff nodules. The lungs exhibited the characteristic findings of long-standing chronic passive congestion. Extensive pulmonary arteriosclerosis and arteriolar sclerosis were present. Most of the small and medium-sized arteries were involved by a necrotizing angiitis characterized by fibrinoid necrosis of the media and adventitia and an acute inflammatory reaction (Fig. 5). The reacting cells were largely neutrophils. The right main pulmonary artery showed thrombosis, necrosis of the entire wall, and a neutrophilic inflammatory reaction extending into the surrounding tissues.

Case 5

K. S. S. was a white girl, 3½ years old, who was admitted to Children's Hospital, Cincinnati, Ohio (no. 85952), on October 10, 1952, for cardiac catheterization studies. She was born in March, 1949, by a normal delivery following an uneventful pregnancy. Neither cyanosis nor cardiac murmurs had been detected at birth. In January, 1952, during a bout of pneumonia, she became cyanotic. Following electrocardiographic and roentgenographic studies, she was referred to another hospital, where polycythemia, leukocytosis, and a lowered blood oxygen saturation were dis-

covered. She was treated with a low sodium diet and discharged. Following discharge, she suffered from exertional dyspnea and lethargy. Three weeks prior to admission, cough appeared and cyanosis increased.

On admission, temperature was 100° F. The pulse was 120 to 150; respirations, 30 to 40; blood pressure, 140/80 mm. of Hg. The child was pale and cyanotic, and there was congestion of the mucous membranes and a bulging right anterior chest wall. The lungs were clear to percussion and auscultation. The heart was enlarged, and sinus tachycardia was present. A systolic thrill and a murmur were detected over the precordium, both in the pulmonic area and in the cardiac apical region. Laboratory findings on admission included a hemoglobin value of 16.2 gm. per 100 cc. and a white blood cell count of 11,650 per cmm., with 75 per cent neutrophils. By October 15, 1952, the white blood cell count was 18,200 per cmm. The urine was not remarkable, and a blood culture was negative. Electrocardiogram revealed evidence of right ventricular hypertrophy. Fluoroscopic and roentgenographic examination of the heart demonstrated right ventricular enlargement and excessive pulsation of the main pulmonary arteries.

Treatment consisted of digitalis, mercurial diuretics, penicillin, and oxygen therapy. On the day after admission, the temperature rose to 103° F., and the edge of the liver became palpable. Dyspnea increased, the pulse slowed, and the patient expired on the sixth hospital day.

Necropsy (C-52-88) was performed 21 hours after death. The child appeared under-developed, and cyanosis and clubbing of the fingers were noted. The significant gross findings were limited to the thoracic viscera. The enlarged heart weighed 135 gm. Its apex extended to the left anterior axillary line. Section revealed a trilocular heart resulting from total absence of the interatrial septum. The left ventricle was hypertrophied and dilated, its wall measuring 6 to 7 mm. in thickness. The left ventricular wall measured 5 to 6 mm. in thickness. The pulmonic ring was dilated and the pulmonary arteries showed intimal atherosis. The lungs weighed 100 gm. each. The right lower lobe contained a solitary fibrocaseous nodule measuring 1 cm. in diameter. Both lower lobes had increased consistency and were hyperemic and friable. The remaining portions of the lungs were congested and edematous. The pulmonary arteries were dilated. The purplish red hilar lymph nodes were enlarged and one contained a caseous nodule. Other necropsy findings were non-contributory.

On microscopic examination of the heart, moderate interstitial edema and slight epicardial fibrous thickening were found. The lungs were congested and contained focal hemorrhages. Severe sclerosis of arteries of all sizes, and of arterioles, had proceeded to obliteration in some areas. Several medium-sized arteries contained organizing mural thrombi. In many, there was extensive mural fibrinoid necrosis, usually excentrically placed, and a severe inflammatory reaction extended into the adventitia (Fig. 6). The predominant reacting cells were neutrophils. In other arteries, the lesion had proceeded to scarring of the

wall, aneurysm formation, and canalization of intraluminal thrombi (Fig. 7). The caseous lesions described grossly were localized areas of granulomatous inflammation, probably tuberculous. However, stains for acid-fast bacilli were negative. Sections of liver, spleen, ileum, esophagus, stomach, adrenal gland, kidney, tibia, vertebra, and rib exhibited no findings related to the major disease process. The vascular components of these structures were normal.

Case 6

M. B., a 16-year-old, white female, consulted a physician on November 26, 1954, with complaints of exertional dyspnea and fatigability of 1 month's duration. Physical examination revealed poor nutrition, pulse of 96, and blood pressure of 112/90 mm. of Hg. Cyanosis confined to patchy areas on the skin of the chest and legs was noted. Examination of the chest revealed slight cardiomegaly and a loud second heart sound in the pulmonic area. A harsh, musical, systolic murmur could be heard best in the fourth intercostal space to the left of the sternal border. Minimal ankle edema was present. Roentgenograms of the heart showed enlargement of the pulmonary conus and cardiomegaly, apparently due to right ventricular enlargement. Electrocardiograms indicated right ventricular hypertrophy.

The patient was believed to be suffering from some form of congenital cardiac anomaly, and it was planned to admit her to the hospital for further investigation. However, on December 6, she had an exacerbation of congestive heart failure, characterized by increasing ankle edema and dyspnea. On the following day the dyspnea became severe. Early on the morning of December 8, 1954, the patient died at home.

Necropsy (N-54-597) was performed at the Cincinnati General Hospital less than 6 hours after death. Externally, significant findings included evidence of poor nutrition, cyanosis of the skin and mucous membranes, and edema of the legs. The pertinent gross findings were limited to the thoracic contents, the balance of this complete necropsy revealing only severe passive congestion of all viscera. The lungs were normal in size, the right lung weighing 205 gm. and the left, 175 gm. In both lower lobes there were poorly defined grayish areas of nodularity measuring up to 4 cm. in diameter. These were firm and, on cut section, were not well demarcated from the surrounding parenchyma. Aside from a moderate degree of atherosclerosis of the major pulmonary arteries, the lungs were otherwise unremarkable. The heart was enlarged, weighing 390 gm. The right ventricle was markedly hypertrophied, measuring 8 mm. in thickness, while the left ventricle measured 10 mm. in thickness. The valves, myocardium, and coronary arteries appeared normal. No congenital anomalies of either the heart or great vessels were noted.

Microscopic examination of the heart revealed focal fragmentation of muscle fibers and perivascular fibrosis. The major microscopic findings were encountered in the arteries of the lung, the pulmonary pa-

renchyma showing only patchy atelectasis and alveolar emphysema. About half of the smaller pulmonary arteries were occluded by old or relatively recent miliary thrombi. Many of the older occlusions were canalized, while others were manifested by organizing miliary excrescences projecting into the lumen of the affected vessels. The more recent occlusions contained residual fibrinous deposits in varying stages of organization. Arteries and arterioles of similar size and containing no thrombi revealed severe sclerosis characterized, in the main, by muscular hyperplasia, intimal proliferation, and luminal narrowing. The major pulmonary arteries revealed atheromatous areas. In the right middle lobe a small artery showed, at the point of its bifurcation, massive mural fibrinoid necrosis and inflammatory reaction (Fig. 8). The reacting cells were largely neutrophils and the inflammatory process extended into the perivascular stroma. Aside from passive congestion, no other significant microscopic findings were noted in other organs. Sections of aorta, heart, liver, spleen, pancreas, adrenal glands, kidneys, parathyroid glands, thymus, lymph node, ovary, and bone revealed no evidence of vasculitis.

Discussion

In all the cases described, the pathologic changes are consistent with those of periarteritis nodosa as originally described by Kussmaul and Maier.[1,3-6] They are identical morphologically with those described in the systemic form of the disease with respect to (1) the histologic appearance of the lesions, (2) the size and type of the vessels involved (small and medium-sized arteries), (3) the coexistence of acute and healing lesions in the same case, and (4) the tendency for the changes to occur at points of bifurcation of arteries and to affect only a portion of the circumference of the involved vessels.

Worthy of note is the fact that all were associated with both clinical and morphologic evidences of pulmonary hypertension. The lesions were limited to pulmonary arteries and were not associated with a contiguous pneumonitis.

In the reviews and individual reports of periarteritis nodosa in the medical literature, there has been variation in the recorded frequency of pulmonary involvement. This has been a direct result of the controversy concerning the pathogenesis of the disease and the histologic criteria for its diagnosis. One group of investigators has suggested that this disease is a complication of hypertension,[4,6,7-12] a second group has held that it is a manifestation of hypersensitivity,[13,14] and the two have disagreed as to the histopathology.

Actually, if the morphologic criteria which have been enumerated are applied strictly, authentic reports of pulmonary periarteritis nodosa are uncommon. Association with the Eisenmenger complex and with pulmonary hypertension has been noted by Old and Russell[15,16] and was present in one of the 2 cases described by Kipkie and Johnson.[17] In both, there was limitation of the arteritis to the pulmonary vessels. Pulmonary periarteritis nodosa has been reported in one of 3 necropsied cases of patent ductus arteriosus with pulmonary hypertension (Hultgren *et al.*[18]), in association both with mitral stenosis and with cor pulmonale of unknown etiology (Symmers[19]), and with pulmonary arterial and arteriolar sclerosis of unknown etiology (Kirshbaum,[20] Eskelund,[21] Berthrong and Cochran[22]). McKeown[23] found 4 instances in 111 cases with cor pulmonale collected from a series of 6,770 necropsies. Lesions were found in the lungs of one of the cases reported by Jones[24] (case 13), but microscopic examination of this case was limited and it is not clear whether pulmonary hypertension was present. A case described by Friedberg and Gross[25] (case 1) appears to be one of periarteritis nodosa limited to the lungs. Although the heart is inadequately described in the necropsy report, the patient had rheumatic mitral disease with a mitral diastolic murmur and was in severe congestive heart failure.

On the other hand, Parker and Weiss[2] found no true angiitis in their series of cases of mitral stenosis with chronic passive congestion of the lungs. However, they did find 5 examples of pulmonary arteriolar necrosis simulating the systemic lesions of accelerated arteriolosclerosis.

On close scrutiny, many other reports of necrotizing angiitis of the lungs fail to meet the criteria listed. Therefore, it would appear to be preferable, for the present, to classify these on a morphologic basis as necrotizing angiitis rather than as periarteritis nodosa.[26] Zeek[5,6] has proposed the term hypersensitivity angiitis for a form of necrotizing angiitis characterized by widespread involvement of arterioles, venules, and capillaries, both pulmonary and systemic, and usually associated with a history of allergic sensitization. Unlike periarteritis nodosa, the lesions have a tendency to be in the same phase of development in a given instance. According to Zeek, the angiitis associated with rheumatic fever is morphologically identical. This form of necrotizing angiitis has been referred to also as "the microscopic form of periarteritis nodosa" by Godman and Churg.[27] The choice of each of these terms is, perhaps, unfortunate, in view of the uncertainty concerning the etiology of the various forms of necrotizing angiitis. However,

these characterizations present a convenient means of classifying the angiitides on the basis of histologic appearance.

Examples of hypersensitivity angiitis, since the report of Ophüls,[28] frequently have been called periarteritis nodosa. The cases of Cohen, Kline, and Young,[29] Spiegel[30] (case 13), and Bayley, Lindberg, and Baggenstoss[31] appear to fall into this category. The pulmonary arteritis in an individual with systemic periarteritis nodosa illustrated by Harkavy[32] (case 14, Fig. 4) suggests that the lesions in the lung are those of hypersensitivity angiitis. The coexistence of both forms of angiitis has been reported previously.[7] More, McMillan, and Duff[33] and Black-Schaffer[34] have described similar cases of angiitis in the lungs, associated with sulfonamide hypersensitivity.

In 1951, Churg and Strauss[35] described a group of cases of allergic granulomatous angiitis in which pulmonary involvement was common. This disease may be differentiated morphologically from periarteritis nodosa by early exudation of eosinophils and later formation of granulomas containing giant cells. In their review of Wegener's granulomatosis, Godman and Churg[27] cited numerous examples of this type of angiitis in the medical literature, many reported under the diagnosis of periarteritis nodosa.

To classify many other cases reported as periarteritis nodosa is impossible on the basis of the morphologic data provided by the authors. Gruber[36] gave the incidence of pulmonary involvement as 3.7 per cent in his large series, but provided insufficient data to verify the authenticity of the diagnoses. Harris, Lynch, and O'Hare[37] noted a 25 per cent incidence of periarteritis of the lungs in their review of the literature, but many of these reports are based entirely upon clinical evidence. In view of the frequency of the occurrence of eosinophilia and bronchial asthma in this group, it seems probable that some may have been examples of either "hypersensitivity" or allergic granulomatous angiitis. Rothstein and Welt[38] reported a case of their own and found two other examples of pulmonary periarteritis in children in their review. Their patient had a rheumatic mitral lesion and showed evidences of cardiac failure, but the morphology of the lesions is inadequately discussed to permit evaluation. This is also true of the series of cases reported by Wilson and Alexander,[39] many of which were believed to have pulmonary lesions. In this series, there was an 18 per cent incidence of bronchial asthma.

The impossibility of recognizing pulmonary involvement in periarteritis nodosa with any degree of certainty by clinical means is illus-

trated by Harkavy.[40] In this series of 8 cases with pulmonary infiltrates, there was one in which cutaneous biopsy revealed a lesion resembling periarteritis nodosa, but subsequent necropsy failed to demonstrate pulmonary angiitis. Felsen[41] reported the diagnosis of periarteritis nodosa by sigmoidoscopic biopsy on two occasions. One of these individuals manifested evidence of both pulmonary and systemic involvement, and, in addition, right-sided cardiac enlargement. No adequate pathologic proof of the identity of the pulmonary lesions is offered. The case of pulmonary periarteritis nodosa in association with systemic angiitis reported by Sandler[42] is inadequately described to permit its evaluation. Douglas *et al.*[43] described a case of patent ductus arteriosus with pulmonary hypertension that manifested lesions in small pulmonary arteries suggestive of, but not diagnostic of healed periarteritis nodosa. In Cabot case 25141,[44] the arterial lesions are not described, but the circumstances suggest that the case may have been one of allergic granulomatous angiitis.

In those reported cases in which the presence of pulmonary periarteritis nodosa has been substantiated by clear-cut evidence, invariably there have been both clinical and pathologic features indicating the presence of pulmonary hypertension, and the pulmonary arteries have been the only vessels involved. This is true also of the 6 cases reported here. In view of the controversy concerning the relationship of this disease to systemic hypertension and the infrequency of pulmonary arterial involvement in periarteritis nodosa, the simultaneous occurrence of pulmonary hypertension and periarteritis nodosa limited to the lungs appears to be a noteworthy phenomenon. Unfortunately, however, the number of recorded cases currently available is still too few to warrant a conclusion that this is an invariable circumstance.

Summary and Conclusions

Six cases of necrotizing angiitis are reported in which the vascular lesions were limited to the lungs, associated with prolonged pulmonary hypertension, and met the morphologic criteria for the diagnosis of periarteritis nodosa.

Three of these examples were associated with advanced mitral stenosis; one was associated with congenital absence of the interatrial septum; in the fifth, pulmonary hypertension was of unknown etiology, and was accompanied by pulmonary arterial and arteriolar sclerosis; in the sixth case, pulmonary hypertension was associated with multiple miliary thrombotic occlusions of small pulmonary arteries.

From these 6 cases and from a resumé of other cases of pulmonary necrotizing angiitis in the literature, criteria for the diagnosis of periarteritis nodosa and other forms of necrotizing angiitis have been formulated and are discussed.

It is concluded that the common association of pulmonary hypertension with this type of localized arteritis suggests a pathogenetic relationship.

I wish to express my appreciation to the Departments of Pathology of Bethesda Hospital and Children's Hospital, Cincinnati, Ohio, for permission to use material from their files.

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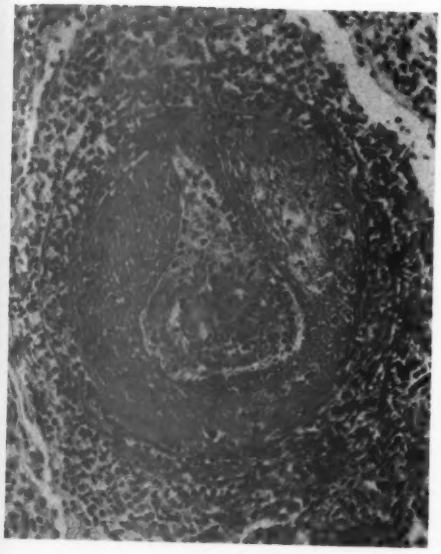
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 1. A medium-sized pulmonary artery with massive fibrinoid mural necrosis and extensive inflammatory infiltrate, consisting largely of neutrophils. Hematoxylin and eosin stain. × 160.

FIG. 2. Case 2. A small pulmonary artery exhibits total destruction of its wall and extensive mural and perivascular inflammation. Hematoxylin and eosin stain. × 160.

FIG. 3. Case 2. A large pulmonary artery shows marked sclerosis and fibrinoid alteration in the intima. Inflammatory infiltrate is largely histiocytic. Media and adventitia appear intact. Hematoxylin and eosin stain. × 80.



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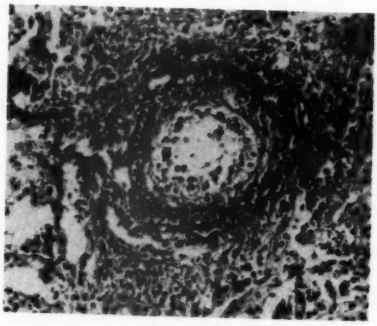

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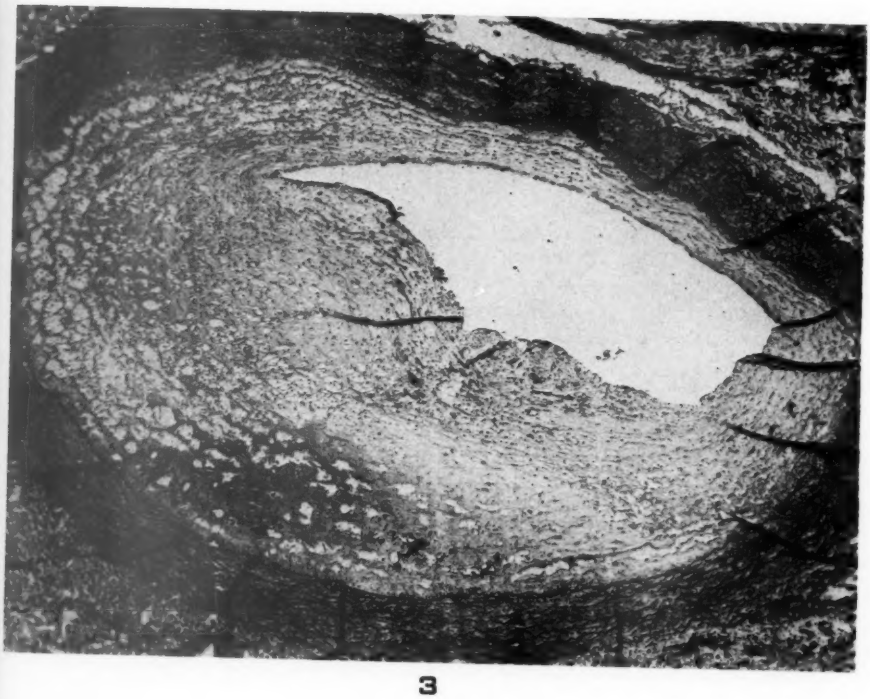

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FIG. 4. Case 3. A large segment of the wall of a small pulmonary artery shows fibrinoid necrosis. There is beginning inflammatory exudation and a thrombus is commencing to form. Hematoxylin and eosin stain. × 650.

FIG. 5. Case 4. Extensive fibrinoid necrosis of the wall of a medium-sized pulmonary artery. The predominant reacting cells are neutrophils. Hematoxylin and eosin stain. × 160.

FIG. 6. Case 5. A segment of a medium-sized pulmonary artery reveals fibrinoid necrosis and mural and perivascular inflammatory exudates. The remainder of the wall is not involved. Hematoxylin and eosin stain. × 160.

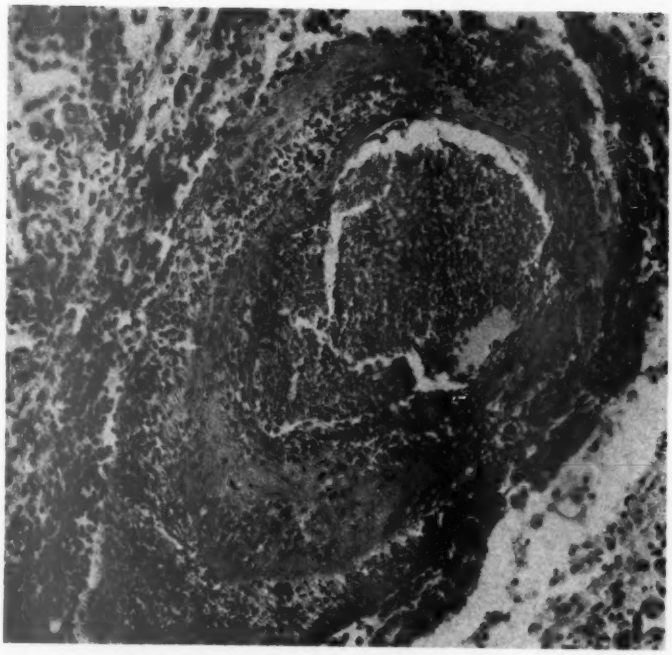

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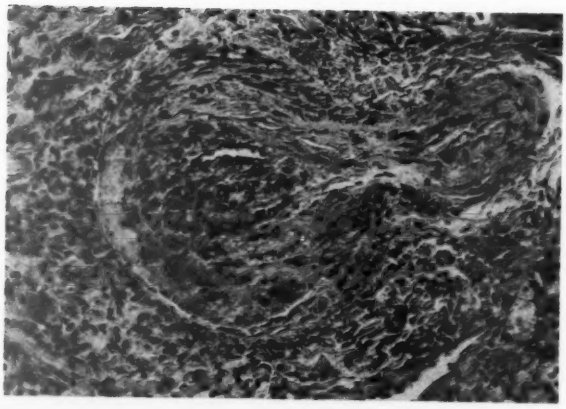
FIG. 7. Case 5. A small pulmonary artery shows severe mural scarring with formation of a micro-aneurysm. There is thrombosis with organization and recanalization. Scattered inflammatory cells are still present. Hematoxylin and eosin stain. × 160.

FIG. 8. Case 6. A small pulmonary artery demonstrating, near its bifurcation, massive mural fibrinoid necrosis and neutrophilic inflammatory reaction extending into periarterial stroma. Hematoxylin and eosin stain. × 160.



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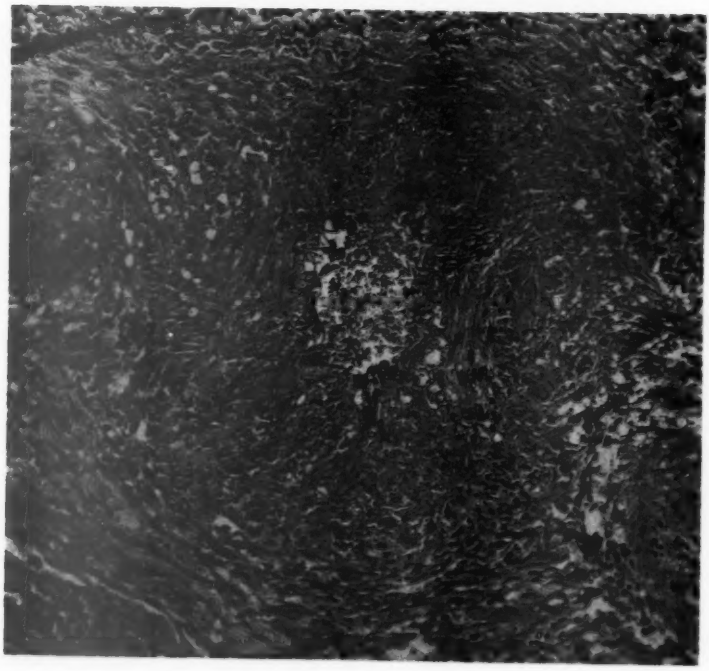

8

EXPERIMENTAL MONILIASIS IN MICE *

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It is generally recognized that *Candida albicans* is the only species of Candida known to be pathogenic to man and to a number of laboratory animals. However, there is little available description of the pathologic changes encountered in experimental moniliasis in animals. In the past, inflammatory reaction to the organism has been attributed either to direct tissue irritation or to thrombosis subsequent to vascular damage by the organisms or a hypothetic toxin.

Redaelli[1] found that intravenous injection of *C. albicans* resulted in rapid diffusion in guinea-pigs, rats, and rabbits, and, that the kidney was the most altered organ although its medulla was rarely involved. He also recorded involvement of the liver, brain, heart, spleen, adrenal glands, and mesenteric lymph nodes although no description of the pathologic alterations was recorded. Redaelli believed that the lesions were of embolic character, the organisms comprising the emboli.

Stovall and Pessin[2,3] disagreed with this concept and contended that embolism was not the pathogenetic mechanism of systemic moniliasis. They indicated that there were species of Candida much larger in diameter than *C. albicans* which were not pathogenic to laboratory animals. They further demonstrated that septicemia in animals infected with *C. albicans* was always present. This they failed to demonstrate with other species of Candida. It was concluded that *C. albicans* was a very virulent species capable of long survival in animal tissues and, therefore, capable of producing lesions.

Fuentes, Schwarz, and Aboulafia[4] have shown that the rat, rabbit, guinea-pig, and mouse were susceptible to *C. albicans* in the order indicated. They noted in their histologic studies that the lumina of capillaries contained blastospores and pseudohyphae. These were associated with desquamation of endothelium and diapedesis of red blood cells. They suggested the possibility of local toxic damage to endothelium as a mechanism for pathogenicity.

Salvin, Cory, and Berg[5] showed that the use of mucin as a vehicle for the injection of *C. albicans* permitted the employment of relatively smaller numbers of organisms which produced higher mortality in a

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shorter period of time. More recently, Salvin[6] proved that cells of *C. albicans* contain a soluble endotoxin. He demonstrated that filtrates from suspensions of the organisms produced the same lesions as live organisms.

Scherr,[7,8] in his study of the use of yeast cells to enhance the virulence of *C. albicans* for mice and in his subsequent work on the effect of cortisone on moniliasis in mice, used gross pathologic changes in his observations. Many other workers have described isolated organic lesions in case reports.[9-13]

In this paper, we will endeavor to show the distribution and nature of lesions in experimental moniliasis in mice.

Materials

Four groups of mice were studied in addition to a control series. The mice were all white males of the same strain and weighed approximately 20 gm. They were obtained from the same dealer and were reared under identical conditions. The animals were obtained in three batches at different times. The first batch was used in group I and the controls, the second in groups II and IV, and the third in group III. Studies with groups II and IV were carried out 1 year after study of group I was begun. The group III study was carried out a few months after groups II and IV were started. Groups I, II, and III were inoculated intravenously with an isotonic saline suspension of our *C. albicans* "Fuentes"* grown overnight on Saboraud's dextrose agar. The fourth (group IV) group was inoculated with formalin-killed *C. albicans*. The control group was not given any inoculation.

The difficulty of an exact count presented a problem. Count in a hemocytometer included dead organisms while count in a pour plate reduced the true number of organisms by the presence of clumps which grew in a single colony. Our suspension was made by comparison with tube 3 of McFarland's nephelometer.†

Methods and Results

The *control group* consisted of 10 mice which did not receive an inoculum of *C. albicans*. They were kept separate from the inoculated group for 2 weeks and sacrificed at the termination of that period. All mice were necropsied and organs preserved in 10 per cent formalin. Tissue sections were prepared and stained with hematoxylin and eosin. Organs from the 10 mice of the control group showed no recognizable lesions.

* *C. albicans* "Fuentes" was isolated from a case of vaginal moniliasis.

† Tube 3 of McFarland's nephelometer corresponds to 50,000,000 monilia per cc.

Group I

Group I consisted of 80 mice. All were inoculated intravenously under light chloroform anesthesia with 0.1 cc. of a suspension, this amount containing 100,000 organisms. In the process of injection, the suspension of organisms was agitated constantly to prevent sedimentation. Three mice died instantly after injection, probably as a result of faulty technique. The remaining 77 mice were inspected three times a day during the period of study. Dead animals were removed promptly, necropsied, and all organs preserved in 10 per cent formalin. Tissue sections were stained with hematoxylin and eosin and the periodic acid-Schiff stain.

Table I indicates the survival period following inoculation. All animals died within 13 days. Two died after a period of 18 hours. The average survival was 81 hours.

TABLE I
Survival Results, Group I

Number of mice	Survival
	hrs.
2	18
5	30
12	40
5	50
16	65
6	75
14	90
5	110
3	120
5	144
2	168½
2	310
Total 77	

Of 77 animals examined, brain, heart, lungs, and kidneys were not available in 2; brain, heart, and lungs were not available in 3; and brain alone was not available in 5. These organs had been eaten by other animals in the cage before the dead bodies were removed.

The heart was the seat of myocarditis and abscess formation (Fig. 1). The myocarditis was characterized by focal and/or diffuse interstitial inflammation with lymphocytes, and, to a lesser degree, histiocytes and neutrophils. Focal areas of inflammation usually were fairly well circumscribed. Microabscesses were found singly or in coalescent form in cases with more severe myocardial involvement. Yeast cells and pseudohyphae were present in all lesions and their demonstration was facilitated by examination of sections stained with the periodic acid-Schiff stain (Fig. 2). They were in large numbers, particularly in animals which died from 18 to 40 hours after inoculation. These lesions were found in increased number and extent in inverse proportion to survival period. In the 9 animals which survived longer than 144 hours, only 2 had minimal focal myocarditis.

Granulomatous lesions in the heart were seen on two occasions in animals which survived up to 65 hours. These were well circumscribed,

non-caseating nodules made up of histiocytes and bordered by a zone of lymphocytes. No organisms were found in the granulomatous lesions.

The kidneys contained many isolated and coalescent abscesses, predominantly among animals which succumbed between 50 to 120 hours after inoculation. Organisms were present in large numbers in these lesions. In several instances, cortical abscesses had broken through the renal capsule to produce perinephric abscesses (Fig. 3). The abscesses were found regularly in the renal cortical areas of animals surviving less than 120 hours and were much less common in those living longer than 140 hours. When present in the latter group, they were located invariably in the medulla. Three of these, indeed, did not have abscesses at all. In addition to renal abscess formation, all 77 cases exhibited a monotonous pattern of focal or mild diffuse interstitial infiltration of lymphocytes and histiocytes. As with the abscesses, the extent of the reaction was greater in animals which succumbed early and less in animals which survived longer. In the latter group, the interstitial lesions appeared in medullary areas and frequently were associated with pyelitis. In the pelvis of the kidneys in which inflammation was present, numerous organisms were found also in yeast form and as pseudohyphae in calyces, mucosa, and tips of renal pyramids.

Granulomatous lesions of the kidneys were met with regularity among animals surviving 50 to 120 hours (Fig. 4). Fifty-six of the 73 animals examined showed tubercles of recent development. They were generally non-caseating microtubercles consisting of histiocytes concentrically arranged and surrounded by a zone of lymphocytes. They were located both in cortical and medullary regions. Organisms were not demonstrated in the granulomatous lesions.

Lesions of the brain appeared early but were most extensive between 50 and 120 hours. They consisted of foci exhibiting glial cell degeneration and localized infiltrations with lymphocytes and histiocytes (Fig. 5). Organisms were present in many of these lesions.

The spleen in 50 per cent of the animals was the seat of marked histiocytic proliferation in sinusoidal areas and exhibited progression to frank granulomatous formation with central necrosis (Fig. 6). Organisms, ordinarily in the form of yeasts, were noted in these lesions. Animals surviving 50 to 120 hours showed these lesions in greater numbers.

The stomach of the majority of animals surviving less than 65 hours had a superficial mucosal inflammatory process upon which were

superimposed dense plaques or organisms, mostly in the form of pseudohyphae. The organisms overlaid the mucosa and rarely were observed to penetrate the muscular layers. The small intestine, in two instances, exhibited lesions of the same type.

Voluntary muscle from the paravertebral region at the level of the kidneys demonstrated diffuse interstitial histiocytic and lymphocytic infiltration with occasional abscess formation and numerous organisms.

In one instance for each, pancreas, lacrimal gland, prostate, and seminal vesicle were the seats of inflammatory reactions similar to those occurring elsewhere. Organisms were found in these lesions.

Lungs in all animals showed acute congestion, focal hemorrhages, and focal edema. In a few there was non-specific lobular pneumonia. Organisms were never found in the lungs.

The eye, salivary gland, liver, adrenal gland, and urinary bladder failed to show pathologic alterations.

Group II

Group II consisted of 11 mice. These were inoculated with 0.1 cc. of a suspension containing 1,000,000 organisms in the same manner as animals in group I. Two animals were sacrificed 1 hour after inoculation, 2 at 4 hours, 2 at 10 hours, and 1 at 20 hours. The remaining 4 mice died spontaneously between the 10th and 20th hours after inoculation. The mouse sacrificed at the end of the 20th hour was moribund. Heart, lungs, liver, spleen, and brain were fixed in 10 per cent formalin and tissue sections prepared in the same manner as for group I.

Four mice sacrificed 4 hours after inoculation showed no detectable alterations. A few yeasts and pseudohyphae were present in a section of heart from an animal sacrificed after 1 hour. The earliest lesions appeared at 10 hours in the form of interstitial myocarditis, dense hyaline casts in the kidneys, and focal histiocytic proliferation in the liver. In this mouse, also, organisms were seen in the heart. The mice which died between 10 and 20 hours and the last animal sacrificed at the end of 20 hours showed essentially the same pathologic features as described for animals in group I. The lesions were unusually severe.

An unexpected finding in the liver was the presence of focal but widespread interstitial histiocytic proliferation in portal areas or around blood vessels. There were organisms in these lesions. They were found in 7 of 11 animals and were present extensively in the 4 animals which died spontaneously and in the last one sacrificed. These lesions were generally circumscribed and showed no necrosis.

There was lobular pneumonia in 4 mice which died spontaneously. Organisms were present in 2 of the 4 lungs exhibiting pneumonia.

Group III

Group III consisted of 54 mice. These animals were inoculated with 0.1 cc. of a suspension containing 1,000 organisms in the same manner as in groups I and II. Two mice died promptly after injection and were discarded. The remaining 52 mice were sacrificed as follows: 4 mice at 6 hours after inoculation, 4 at 12 hours, 4 at 19 hours, and 3 animals daily until the 15th day.

None of the 52 animals in group III died spontaneously. At necropsy, heart, lungs, liver, kidneys, and brain were preserved in 10 per cent formalin, and tissue sections stained with hematoxylin and eosin and by the periodic acid-Schiff method were prepared.

Thirty-one of 52 animals (59.6 per cent) showed myocarditis of focal or diffuse character, similar to that previously described for groups I and II. Focal myocarditis was recognized in one mouse sacrificed 6 hours after inoculation. This lesion appeared with maximum intensity among animals sacrificed on the fourth day. It was found in all animals up to the ninth day, after which only a single mouse (sacrificed on the twelfth day) had mild focal myocarditis. Only 4 subjects exhibited micro-abscesses in the myocardium and these were encountered among mice sacrificed as early as the first day and as late as the sixth day after inoculation. Two myocardial granulomas were seen, one in an animal sacrificed on the second day and another in one sacrificed on the fourth day. Only two hearts contained organisms. The nature of the lesions was similar to that described in groups I and II but they were less severe.

As in the heart, renal lesions consisting of focal and diffuse interstitial infiltrates with lymphocytes and histiocytes were found as early as 6 hours after inoculation. They appeared regularly among animals sacrificed up to the 13th day, a total of 36 (69 per cent) exhibiting this process. Twelve (23 per cent) of those sacrificed after the fourth day showed abscesses, but organisms appeared in only 2 of the mice sacrificed before the seventh day. From the eighth to the twelfth day, the interstitial inflammation was associated with marked pyelitis, many cellular casts in the tubules, abscesses, and innumerable organisms in the medullary and pelvic regions. Granulomas were found in only 2 animals.

Lesions in the brain were similar to those observed in group I. They were present in 48 per cent and were most common among animals sacrificed from the fourth to the eleventh day. There were abscesses

in 8 cases and organisms in 5 of these. A granuloma was seen only once.

In the liver, the peculiar periportal and perivascular histiocytic infiltrations were observed in all but 6 cases. The 6 animals without this lesion were sacrificed latest in the series (14th and 15th days). There were abscesses in 5 and a granuloma with central necrosis in one. Organisms were encountered in one abscess. The hepatic lesions were similar to those encountered in group II (Figs. 7 and 8).

Group IV

Group IV consisted of 12 mice inoculated intravenously with 0.1 cc. of a suspension containing 1,000,000 dead organisms. The organisms were killed in formalin and washed five times in isotonic saline solution. Pairs of mice were sacrificed at 1 hour, 4 hours, 10 hours, 20 hours, 2 days, and 5 days after inoculation. None of these animals died spontaneously. As in groups II and III, sections were prepared from heart, lungs, spleen, kidneys, and brain.

There were no recognizable pathologic alterations in the heart. Six animals had focal lymphocytic infiltrations in the kidneys and 4 had similar lesions in the brain. The liver in all cases had histiocytic proliferation similar to that found in groups II and III. However, no organisms were demonstrated.

Discussion

It was apparent from group I that mice succumbed at an average of 3 to 4 days after intravenous inoculation with 100,000 organisms *(C. albicans)*. Renal lesions in the form of interstitial inflammation, abscesses, and granulomas were present in all cases. The intensity of the renal process suggested that all animals might have succumbed without associated lesions in other organs. However, in view of the coexistent myocarditis and encephalitis, most prominent in animals which died early, it seemed likely that early death was brought about by a combination of all the lesions.

There is no ready explanation for the predominance of lesions in the heart, kidneys, and brain, nor for the absence of hepatic changes in group I. Special organ predilection has been observed with other microorganisms. In point, *Histoplasma capsulatum* often localizes in the liver and rarely in the kidney. It was interesting to note that in the presence of considerable kidney damage, the urinary bladder remained free of lesions. *Candida albicans* grows best in a slightly acid medium (pH 5.0). Urine of rodents has a very alkaline reaction and this may have inhibited the growth of organisms in the bladder.

Inflammation and abscesses in voluntary muscles adjacent to the kidneys were probably the result of local extension from cortical abscesses of the kidney which had broken through the capsule. The hematogenous route of localization cannot, of course, be excluded.

The greater number of granulomas encountered among animals which succumbed later in group I would indicate that such a lesion is a manifestation of chronicity or indolence. This, however, did not prove to be the case in group III, in which granulomas occurred with much less frequency. We believe that in the latter study the number of organisms introduced was insufficient to incite granulomatous lesions.

Group II demonstrated that the intravenous inoculation of a heavy dose of *C. albicans* in mice resulted in rapid fatality (within 24 hours). Judging from the nature and extent of the lesions observed in the 4 mice which succumbed between 10 and 20 hours, it seems reasonable to conclude that the other animals in the series would have expired within 24 hours had they been permitted to live. In fact, the mouse sacrificed at 20 hours was moribund.

This method might present a speedy means of determining the pathogenicity of certain species of Candida. The intravenous introduction of a suspension of an unknown Candida would be an easy and inexpensive procedure.

The presence of a considerable number of circumscribed collections of histiocytes accompanied by demonstrable organisms in the liver was an unexpected occurrence since this had not been encountered in any animals in group I. This phenomenon prompted us to proceed with studies in group III and IV. We believed originally that hepatic lesions were the result of massive inoculation. However, since 46 of 52 animals (88.5 per cent) in group III showed the same lesions, although without demonstrable organisms, this was obviously not the case. Indeed, 5 animals showed hepatic abscesses and 3 showed granulomas. Moreover, focal histiocytic proliferation also appeared in group IV. At present there is no ready explanation for the variation of response in the liver.

A small inoculum of *C. albicans* has proved to be incapable of producing fatalities in mice. Lesions of mild nature have been produced, however.

The mechanism of death in mice with intravenous inoculation of *C. albicans* would appear to be attributable to toxemia. This is in keeping with Salvin's[6] demonstration of an endotoxin in *C. albicans*. It is not possible, however, to rule out myocardial damage as an im-

mediate cause of death since the cardiac lesions were extensive and severe. This was especially true with a heavy inoculum. It does not seem reasonable to consider vascular occlusion from embolization of fungi an explanation for the pathogenesis. In all sections examined, only one artery contained a mycotic occluding embolus.

Summary

Four studies were made to determine the pathogenicity of *Candida albicans* "Fuentes." Group I consisted of 80 mice inoculated intravenously with 0.1 cc. of a suspension of *C. albicans* (containing 100,000 organisms). All animals succumbed within 13 days. Two died as early as 18 hours. The average survival period was 3½ days.

Group II consisted of 11 mice inoculated with 0.1 cc. of a suspension of *C. albicans* (containing 1,000,000 organisms). This proved fatal to 4 animals within 20 hours. From the nature of the lesions found in the remaining 7 animals, we assumed that all would have died spontaneously within a day if the animals were not sacrificed earlier.

Group III consisted of 54 mice inoculated intravenously with 0.1 cc. of a suspension of *C. albicans* (containing 1,000 organisms). No fatalities resulted.

Group IV consisted of 12 mice inoculated intravenously with 0.1 cc. of a suspension of killed and washed *C. albicans* (containing 1,000,000 organisms per ml.). No fatalities resulted after 5 days and no lesions comparable with those produced in groups I, II, and III were encountered.

Experimental moniliasis in mice was manifested by a disseminated disease characterized by interstitial inflammation, abscesses, and formation of granulomas. These lesions were most prominent in the heart, kidney, brain, and spleen. Hepatic changes were variable. Eyes, salivary glands, adrenal glands, urinary bladder, and lungs failed to show pathologic alterations directly attributable to the infectious agent.

Myocarditis appeared to be the earliest manifestation of the disease and probably was the immediate cause of death in the majority of animals in groups I and II.

Interstitial nephritis was of constant occurrence. Medullary localization was prominent among animals which survived for the longer periods.

Encephalitis was a late manifestation of the disease.

Granulomas, judging from observations in group I, indicated chronicity or indolence.

Survival time and tissue reaction depended largely on the number of inoculated organisms.

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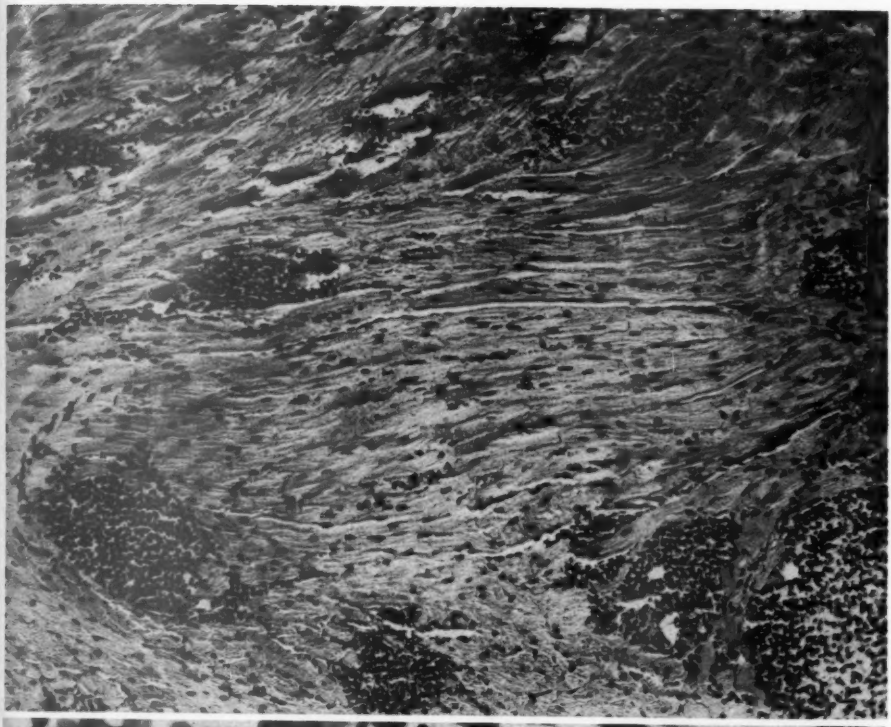
LEGENDS FOR FIGURES

FIG. 1. Heart, from mouse of group II. Interstitial infiltrate of lymphocytes, histiocytes, and neutrophils with abscess formation. × 160.

FIG. 2. Numerous yeast cells and pseudohyphae in a myocardial abscess. Animal from group I which died between 30 to 40 hours after inoculation. × 900.



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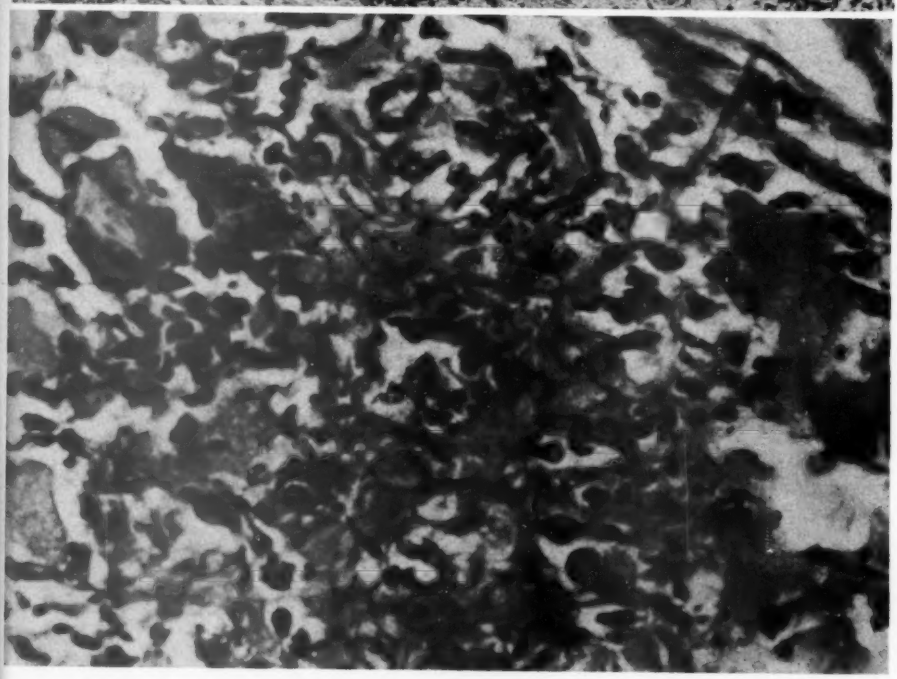

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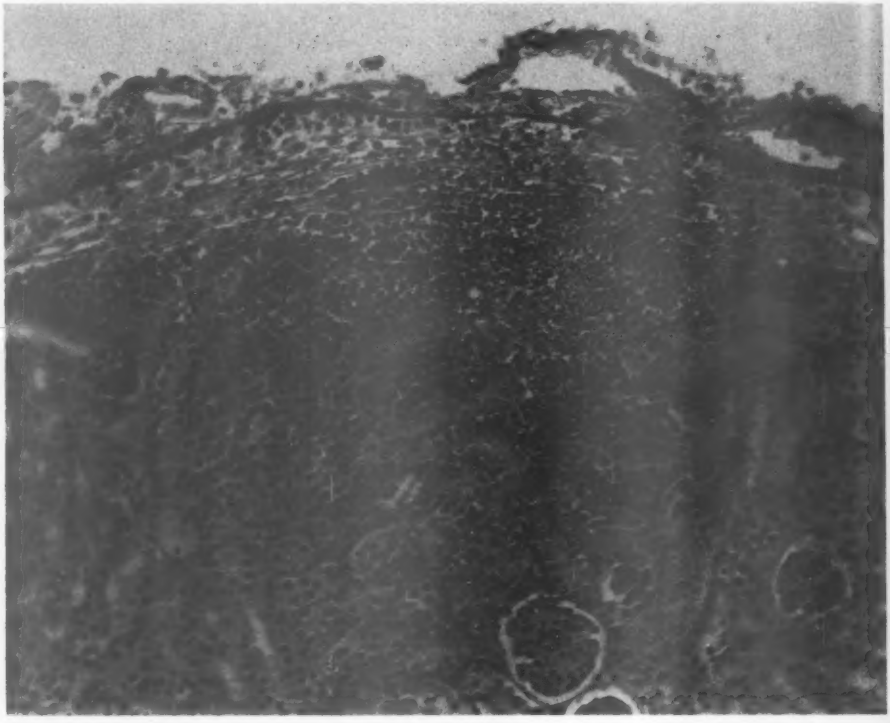
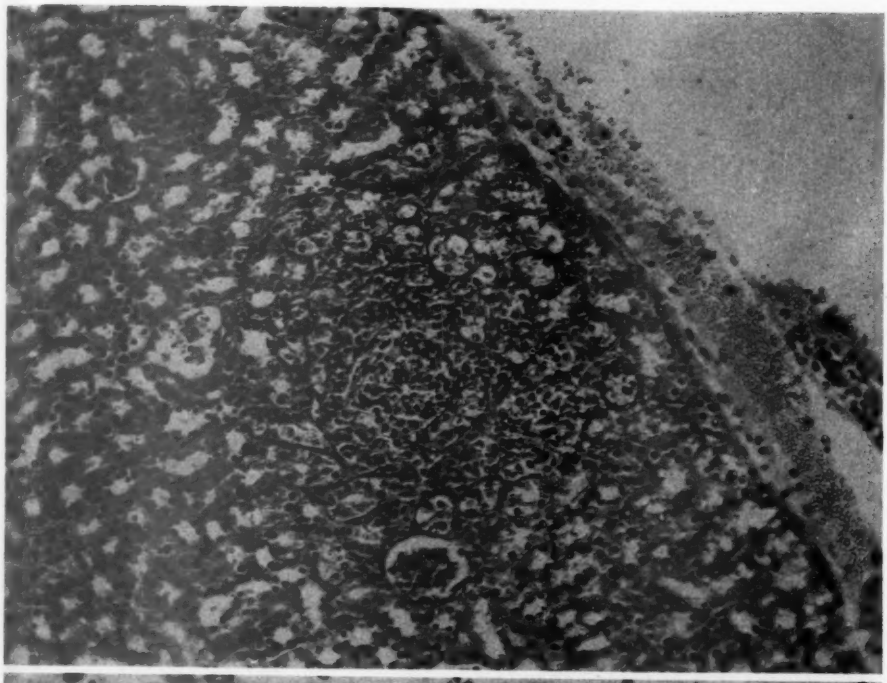


FIG. 3. Kidney, group I. A cortical abscess in an animal which died between 120 and 144 hours. The lesion has broken through the surface, with perinephric abscess formation. × 200.

FIG. 4. A granuloma of recent development in the kidney of a mouse from group III. The lesion consists primarily of histiocytes and lymphocytes. × 170.

FIG. 5. Focal encephalitis (mouse from group I), characterized by degeneration and necrosis of neuronal elements. Infiltrate of histiocytes and lymphocytes. × 650.



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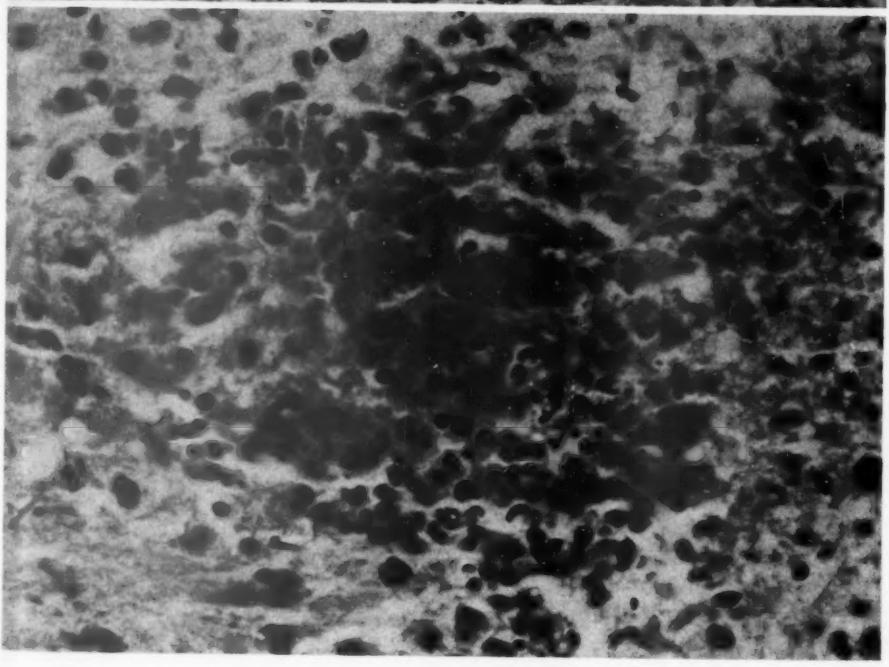

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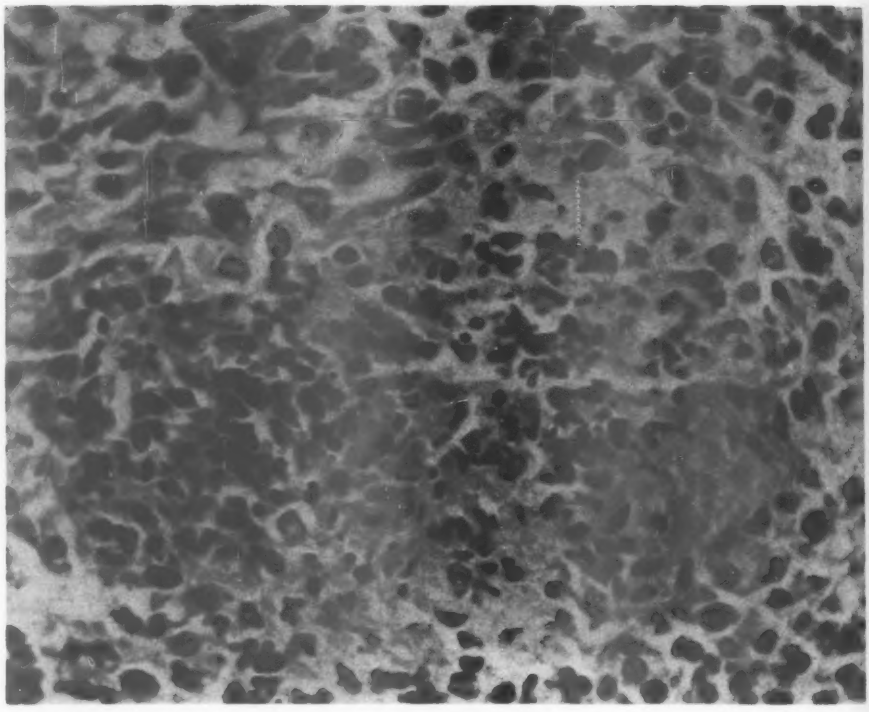
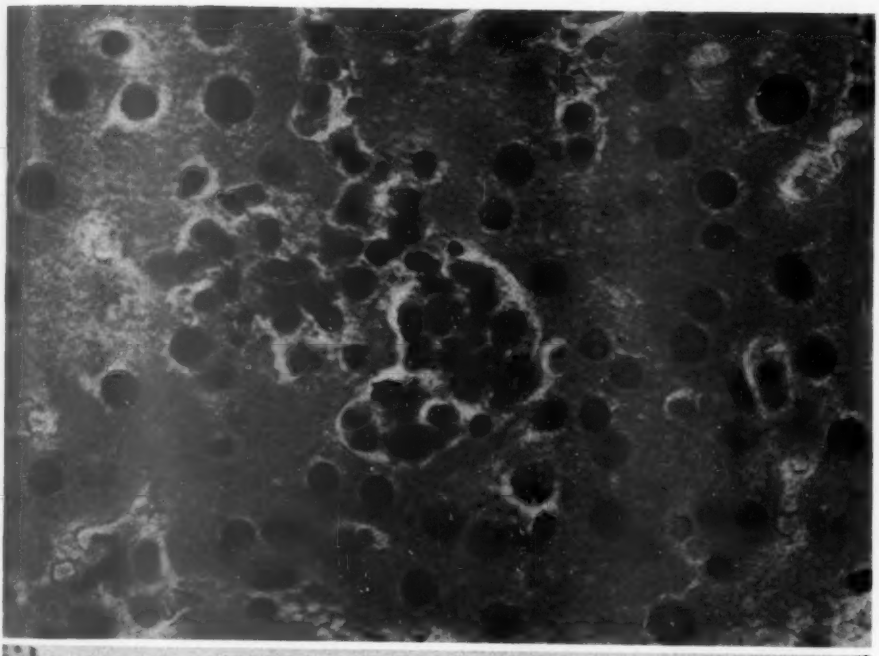


FIG. 6. Two coalescent granulomas of recent development in the spleen (mouse from group I). The reaction is primarily histiocytic, although the lesion on the left shows early necrosis. × 650.

FIG. 7. Liver, group II. Area of histiocytic cell proliferation in sinusoidal space. × 900.

FIG. 8. Liver, group III. Perivascular granuloma with early central necrosis. × 170.



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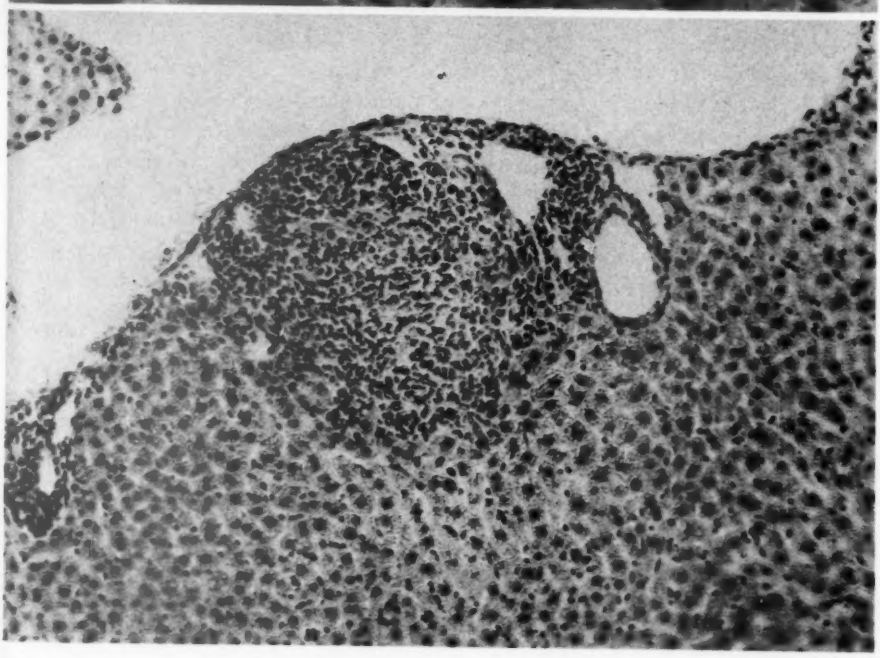

8

STUDIES ON RICKETTSIAL TOXINS

III. Histochemical Survey of Selected Tissue Enzymes in Mice Receiving Murine Typhus Toxin *

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Recent studies have elucidated some of the phenomena which result from the injection of lethal doses of rickettsial toxin into susceptible animals.[1-5] While hemolysis appears to be a prominent finding in the rabbit in acute deaths due to the action of rickettsial toxin,[1] the process in the mouse and rat is characterized by progressive hemoconcentration.[1,2] *In vivo* studies on the mouse have revealed a sequence of vascular reactions including slowing of the blood flow, vasoconstriction, and edema of the skin; and experiments with tracer substances have indicated that progressive leakage of plasma occurs throughout most of the tissues.[3]

It has been a basic premise in the series of studies on rickettsial toxin emerging from this laboratory that the initial, or primary, action of the toxin which sets off the sequence of physiologic disturbances leading ultimately to death of the animal is probably a well defined process and conceivably an effect on some enzyme system. Indeed, some *bacterial* endotoxins have been shown to affect carbohydrate metabolism in rabbits and rats *in vivo* and in liver slices and homogenates *in vitro*.[6-8] Although there is no evidence on hand at this time to suggest that rickettsial toxins and bacterial endotoxins necessarily produce similar responses, it is conceivable that some interference with energy metabolism of this general type might be operative in rickettsial toxemia. Furthermore, such an effect might be limited to specific structures or tissues within the organs and, hence, might not be demonstrable by the conventional methods of enzyme study. Accordingly, histochemical methods, which permit observations of enzyme action in relation to tissue structure, have been employed in the present study on selected oxidizing enzymes of the glycolytic system and the citric acid cycle in mice injected with toxic suspensions of *Rickettsia mooseri*. In addition, the effect of toxin injections on "endogenous metabolism," i.e., the ability of tissue sections to reduce tetrazol in the absence of added substrate, and also on peroxidase, alkaline phospha-

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tase, adenosine-5-phosphatase, and myristoylcholine esterase activities was studied by histochemical methods.

The current studies indicate that the injection of lethal doses of rickettsial toxin into mice does not influence significantly the enzyme reactions tested in the tissues selected for study. An observed decrease in the "endogenous metabolism" in the livers of mice moribund from the action of rickettsial toxin appears to be a non-specific phenomenon.

Materials and Methods

Rickettsial Toxin

Suspensions of the Wilmington strain of *R. mooseri* served as toxin in the present study. The methods of preparation and assay were the same as those described previously.[3]

Injection of Mice and Processing of Tissues

White male mice of the Bagg strain, each weighing 18 gm., were injected intravenously with 0.5 ml. of yolk sac suspensions containing 2 LD_{50} of toxin and were sacrificed by crushing the cervical cord about 2½ hours after the injection. Control animals were injected with heat-inactivated (56° C. for 30 minutes) rickettsial suspensions, but otherwise were treated identically. Following sacrifice of the animal, the desired tissues were removed quickly, and thin blocks were frozen in isopentane chilled with liquid nitrogen. The blocks were stored over solid carbon dioxide until used, which was generally within 24 hours. In the study of liver "endogenous metabolism," mice were sacrificed 1¼ and 2½ hours after injection of the toxin; a control group was subjected to trauma in a Noble-Collip drum.[9]

Brain, heart, liver, lung, spleen, and kidney were all examined by each histochemical method with the following exceptions: (1) brain only was tested by the procedure for myristoylcholine esterase, and (2) spleen, liver, and lung only were tested for peroxidase. Peripheral blood smears also were tested for peroxidase.

Histochemical Procedures

Dehydrogenases. For the demonstration of succinic, lactic, and α-glycerophosphate dehydrogenases, sections 10 or 30 μ thick were cut from the frozen tissue blocks in a cryostat,[10] placed on glass slides coated with a thin layer of glycerol-bovine albumin, air dried for about 5 minutes, and then incubated aerobically for 30 minutes at 37° C. in the appropriate reaction mixtures shown in Table I, in which neotetrazolium chloride was used as a reduction indicator.[11] Succinate,[11,12]

lactate,*[11,12] and α-glycerophosphate[13] have all been employed previously as substrates in the tetrazol procedure. Following incubation, the slides were fixed in 10 per cent formalin and then mounted in glycerol. In some instances they were counterstained with 0.5 per cent aqueous methyl green solution.

The procedures for demonstrating triose phosphate dehydrogenase and "endogenous metabolism" differed from those just described. In both instances, substances soluble in the incubation mixture, and hence subject to dilution by the reagents, are necessary for the reaction. Therefore, in order to obtain reproducible results, it was necessary to employ a minimum volume of the reaction mixture and to maintain a constant ratio between reagent and tissue volumes throughout the study. Hence, tissue sections of constant volume were obtained by the well established quantitative histochemical procedure[14] of punching a cylinder of uniform diameter from a frozen block of tissue and then cutting sections of constant thickness from it. For comparative purposes it was essential to use sections from the same anatomical sites. Each section was then placed in an individual square of piccolyte resin built up on a glass slide and, after allowing the sections to dry, 0.05 ml. of reaction mixture was added to it from a constriction pipette. The reaction mixture for the triose phosphate dehydrogenase was patterned after that of Kun,[15] while for "endogenous metabolism," bicarbonate was included in the mixture (Table I). For studying "endogenous metabolism," sections were used rather than blocks of tissue since the established procedure of incubating such blocks in the tetrazolium solution and then cutting frozen sections may give erratic results due to poor penetration of the tetrazolium salt.[11] Following addition of the reaction mixture, the slides were secured in holders in the horizontal position and placed in a tubular flask fitted with a stopcock at each end. Nitrogen, first bubbled through water, was passed through the flask for about 10 minutes. The flask was then submerged in a 37° C. water bath for the required incubation period. At the end of this time the sections were fixed and mounted.

Peroxidase. A modification of the method described by McJunkin[16] was used for peroxidase staining. Frozen sections were incubated for 5 minutes at room temperature in a 50 per cent ethanol solution containing 0.15 per cent benzidine and 0.03 per cent hydrogen peroxide.

* Lactate oxidation was studied both in the presence and absence of added diphosphopyridine nucleotide and nicotinamide, since the addition of these compounds to the reaction mixture resulted in a neotetrazol reduction pattern different from that obtained when they were omitted.

TABLE I

Histochemical Dehydrogenase Procedures Employed in Studying the Effects of Rickettsial Toxin in Mice

Enzyme	Substrate	Chemical additions*	Gas phase	Characteristics of section employed		Volume of reaction mixture
				Form	Thickness	
					μ	cc.
Lactic dehydrogenase	Sodium lactate, 0.05M		Air	Arbitrary	10 and 30	20–40
Lactic dehydrogenase	Sodium lactate, 0.05M	DPN,† 0.0003M Nicotinamide, 0.03M	Air	Arbitrary	10 and 30	20–40
α-Glycerophosphate dehydrogenase	Sodium glycerophosphate, 0.014M	$NaHCO_3$, 0.025M	Air	Arbitrary	10 and 30	20–40
Succinic dehydrogenase	Sodium succinate, 0.05M	$NaHCO_3$, 0.025M	Air	Arbitrary	10 and 30	20–40
Triose phosphate dehydrogenase	Monomagnesium fructose diphosphate, 0.014M	DPN, 0.0003M Nicotinamide, 0.03M L-histadine HCl, 0.018M	N_2	Circular (6 or 8 mm. diameter)	30	0.05
"Endogenous metabolism"	None added	$NaHCO_3$, 0.025M	N_2	Circular (6 or 8 mm. diameter)	30	0.05

* All reaction mixtures contained 0.25 mg. per cc. of neotetrazolium chloride and 0.1M phosphate buffer, pH 7.4, except for the triose phosphate dehydrogenase procedure in which the pH was 6.9.

† DPN = diphosphopyridine nucleotide.

The sections were washed in water, taken rapidly through alcohol and xylol, and mounted in resin.

Alkaline and Adenosine-5-Phosphatases. The alkaline phosphatase procedure of Gomori[17] and the adenosine-5-phosphatase procedure of McManus, Lupton, and Harden[18] were performed on sections 8 μ thick cut from cold (4° C.), acetone-fixed, paraffin-embedded tissues.

Myristoylcholine Esterase. The procedure of Gomori[19] was followed for myristoylcholine esterase, utilizing frozen sections.

Results

None of the enzymatic reactions studied was influenced to any detectable degree in tissues of mice injected with rickettsial toxin when compared with tissues of mice which had received heat-inactivated toxin. The only effect observed in toxin-treated mice was a decrease in the "endogenous metabolism" of liver sections. This became manifest as a general decrease in the degree of tetrazol reduction over the entire section without any special anatomical localization. The decrease was observed in mice which were moribund about 2½ hours after injection of toxin and, with a single exception, was never found in mice sacri-

TABLE II

Effects of Rickettsial Toxin and of Trauma in the Noble-Collip Drum on the "Endogenous Metabolism" of the Liver of the Mouse

Experimental group	No. of animals	Experimental procedure	Time of sacrifice after experimental procedure	Color of section*	
				Pink	Red
				No. of animals	No. of animals
Uninjected controls	6		*hrs.*	0	6
Injected controls	12	Heat inactivated toxin intravenously	2½	0	12
Rickettsial toxin, midperiod effect	6	Toxin, 2 LD_{50} intravenously	1¼	1	5
Rickettsial toxin, late effect	12	Toxin, 2 LD_{50} intravenously	2½	9	3
Moribund state produced by trauma	7	Noble-Collip Drum, 500 revolutions	5 min.	7	0

* With increasing neotetrazolium reduction, the color sequence is: pink, red, purple, blue.

ficed earlier (Table II). However, the effect on "endogenous metabolism" was not confined to mice receiving rickettsial toxin, for mice moribund from trauma in the Noble-Collip drum also showed decreases in "endogenous metabolism" of the liver which were comparable to those of the terminal phase of toxin-treated mice.

Discussion

The present histochemical study has not disclosed the nature of the primary action of rickettsial toxin in mice nor the site of its occurrence. Unlike Salmonella and meningococcal endotoxins, which have been found to act on certain phases of carbohydrate metabolism,[6-8] the rickettsial toxin, when injected into mice, causes no demonstrable change in a number of enzymatic reactions of the glycolytic system and of the citric acid cycle.

Changes in the "endogenous metabolism" of the liver as suggested by a decrease in the ability to reduce neotetrazolium chloride anaerobically may be interpreted as a non-specific phenomenon, since a similar decrease has been observed in animals which had suffered lethal trauma in the Noble-Collip drum. In mice which have received rickettsial toxin, the terminal state during which the change in "endogenous metabolism" in liver tissue is observed is characterized by pronounced vascular permeability and hemoconcentration.[3] Any metabolic change during that period probably represents a late and secondary phenomenon which would not appear to be related to the direct and primary action of the rickettsial toxin.

The general deterioration of tissues in the near terminal state as evidenced by a diminished "endogenous metabolism" of the liver suggests, in accord with earlier work in this laboratory, that the impressive sequence of secondary effects is largely responsible for the death of the animal.

Summary

A histochemical survey of the following enzymes was carried out in various tissues of mice injected with lethal doses of murine typhus toxin: succinic, lactic, α-glycerophosphate, and triose phosphate dehydrogenases; peroxidase; alkaline phosphatase; adenosine-5-phosphatase; and myristoylcholine esterase. No effect of the rickettsial toxin on the activity of these enzymes was demonstrated.

A late and non-specific decrease in the ability of the liver to reduce neotetrazolium chloride anaerobically in the absence of added substrate was observed, but this is considered an indirect and secondary effect of the rickettsial toxin.

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NEWCASTLE DISEASE VIRUS IN CULTURES OF CHICK EMBRYO TISSUES

Its Multiplication, Titration, and Cytopathogenicity *

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The cytopathogenicity of Newcastle disease virus (NDV) for cultures of chicken embryo tissue has not been emphasized in the literature. Gey and Bang[1] were the first to mention its ability to destroy cells, but reported its action only against chorio-allantoic membrane and skeletal muscle. In the course of investigations using NDV, the cytopathogenic action of the virus for chick embryo heart muscle explants was studied. The infectivity and cytopathogenicity for cells migrating from other chick embryo tissues also were determined. In addition, a tissue culture method was established for reproducible titration of the virus. The growth curve of the virus was determined under certain *in vitro* conditions. The influence of the age of the embryonic tissue, of the amount of outgrowth, and of the amount of tissue on virus multiplication and titration in tissue cultures was determined. Data were collected on the neutralization of virus by immune serum and on the effect of continued passage of the virus in tissue culture on its infectivity.

Material and Methods

Virus. The California no. 11914 strain of NDV, obtained from the American Type Culture Collection, was used in these studies. This virus has an ID_{50} of from 10^{-8} to 10^{-9} in eggs (inoculum of 0.1 ml.), and a hemagglutination titer of 1/320 to 1/1280.

Preparation, Inoculation, Sampling, and Observation of Tissue Cultures. Embryonic chick tissue was obtained from 12 to 14-day-old eggs, unless stated otherwise. The tissues were chopped by a razor blade into fragments measuring 0.5 to 1.5 mm., after which they were cleansed by settling through two tubes, in succession, containing Hanks's salt solution. Thirty to forty fragments were then distributed in a thin layer of chicken plasma (Difco) on the lower third of a 16 by 150 mm. tube. To permit maximum contact of the virus with cells,

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a thin film of plasma was made by allowing the tube to remain in a slanted position for approximately 1 minute and then removing plasma and salt solution. By subtracting the quantity of excess fluid from the amount added to tubes, it was determined that, on the average, the clot consisted of 0.03 ml. of plasma diluted by 0.02 ml. of salt solution. Ten to 15 minutes were allowed for the tissue to adhere to the wall of the test tube, and then 1.5 ml. of a supernatant consisting of the following materials was added:

Chicken embryo extract (EE_{50})*	10%
Horse serum	15%
Fischer's V-614 mixture	75%
Penicillin	100 units per ml.
Streptomycin	100 μg. per ml.
Phenol red	0.004%

* Extract prepared with equal volumes of embryos and distilled water.

In the course of these experiments it was established that the concentration of penicillin and streptomycin had no influence on the multiplication or tissue culture titer of the virus. Tubes were rotated 12 to 14 times per hour in a roller tube wheel at 35° C.

After allowing 2 or 3 days for migration of cells, the supernatant of each tube was completely renewed with an equal amount of fresh medium and 0.1 ml. of virus dilution added. Four tubes were used per virus dilution, except in those cases in which determination of virus multiplication was made. In those instances, enough tubes were inoculated so that the removal of samples did not interfere with the approximate values obtained in 1.5 ml. of supernatant.

The extent of multiplication of NDV in the cultures was determined from the dilution set of four cultures which had received an inoculum of between $10^{1.5}$ to $10^{2.5}$ egg infectious doses per ml. (calculated from the ID_{50} and subsequent dilution of the infected allantoic fluid used for inoculation). At the intervals designated for titration, 0.3 ml. of supernatant was removed from each tube. After centrifugation at 1,500 r.p.m. for 5 to 10 minutes, the material was placed in duplicate glass vials, flame sealed, shell frozen, and stored at dry ice temperature until egg titration was possible. This was usually no longer than 2 weeks after storage.

The ID_{50} of the virus was determined at the same time that the tissue culture titration of the virus was started. Multiplication of the virus was followed in sets of cultures used simultaneously to determine

TC_{50} (see below). To determine multiplication of the virus, a "zero-hour" sample of supernatant, taken from the cultures as soon as possible after inoculation, and a 3-hour sample were titrated to help establish an initial value, although it was realized that rapid adsorption of virus particles might preclude a true estimation. Growth of virus was followed in most of the experiments by assaying samples of supernatant after 1, 2, 3, 4, 5, 7, 9, 12, and 15 days. At these intervals the tubes also were examined for destruction of tissue by the virus. Either no destruction or "complete" destruction finally occurred in the tubes. The destruction occurring in each tube of sets inoculated with the virus dilutions was tabulated to determine a "tissue culture destructive dose, 50 per cent" (TC_{50}) by the Reed and Muench formula.

ID_{50} Titration of Tissue Culture Supernatants. Eight to 10-day-old white Leghorn chicken eggs were used for titration of culture supernatants. These eggs had been incubated at 39 to 40° C. for 7 days and at 35 to 36° C. subsequently. One-tenth ml. of supernatant dilution of each culture, made up in Hanks's balanced salt solution, was inoculated into the allantoic cavity of five eggs. Evidence of infection of the eggs, as tested by hemagglutination, was obtained 3 days after inoculation by mixing 0.5 ml. of allantoic fluid from each egg with 0.5 ml. of 0.5 per cent chicken red blood cells in 12 by 75 ml. tubes. Hemagglutination was interpreted after the tubes had set for 60 to 90 minutes at 4° C., the end point being incomplete agglutination with a definite lentiform center and granular edges. The ID_{50}, as calculated by the Reed and Muench formula, represented the titer of the culture supernatant.

Serial Passage of NDV in Cultures. One-tenth ml. of undiluted, pooled supernatant from a set of 5 to 10 cultures, which had been "completely" destroyed, was used to inoculate another set of 2-day-old cultures. The remaining supernatant from the destroyed cultures was stored at CO_2 box temperatures for subsequent investigations.

Preparation and Assay of Immune Serum. The rabbit immune serum used in this study was prepared by two intravenous injections followed by one intraperitoneal injection of undiluted allantoic fluid ($ID_{50} = 10^{-9.0}$) spaced 10 days apart. Using 8 hemagglutinating units of antigen, the hemagglutination-inhibition titer of the serum was 1:32 in contrast to normal, pooled rabbit serum which had a titer of 1:4. When equal quantities of undiluted immune serum and virus dilutions were incubated at 37° C. for 1 hour, the ID_{50} was reduced from $10^{-8.8}$ to $10^{-5.8}$ (control with normal, pooled rabbit serum).

Results

Appearance of Cells Destroyed by NDV. Regardless of the type of tissue used, the first changes which could be attributed definitely to virus action occurred after 36 hours. In most instances 4 days were necessary for complete degeneration, but this varied from 3 to 7 or 8 days, depending on the strength of the virus inoculum.

The first changes in fibroblasts of the heart and skeletal muscle consisted of a loss of the usual spindle shape of the cells. They became rounded and showed a tendency to clump (cf. Figs. 1 and 2). Next, there was vacuolization and increasing granularity of cells near the explant (Fig. 3). This was gradually followed by more intense granularity which finally resulted in clumps of débris-like material (Fig. 4). It was interesting that these changes usually occurred first in cells close to the explant; however, all cells in the culture were eventually affected. There were occasional cultures in which the outlines of some fibroblasts persisted, but there was a distinct lack of the usual intracellular details, indicating severe damage or death to the cells. No cultures were observed in which only some of the cells appeared destroyed, whereas others remained normal. This was of particular importance in determining a "TC_{50}." The same end result was obtained in cultures of chick embryo brain, liver, lung, and intestine. An example of the changes induced in cultures of intestine is shown in Figures 5 and 6. In liver cultures, the cells, which possessed many of the characteristics of epithelial cells, usually showed marked degenerative changes 1 day sooner.

Titration of NDV by the Roller Tube Technique. Because of the striking difference between infected and non-infected cultures, it was easy to determine the end point of the virus titration. Unless stated otherwise, embryonic chicken heart was used for titrations. To ascertain that virus was not present in undestroyed cultures, some cultures which showed no degenerative changes and which were inoculated with one dilution higher than the end-point dilution of virus were tested by egg inoculation and found to be sterile. In every experiment it was possible to calculate readily the TC_{50} due to the extent of destruction in cultures containing virus.

When the same stock virus suspension was used in ten experiments (see Preparation, Inoculation, etc.), the geometric mean of the TC_{50}'s was $10^{-7.7}$, with a range of $10^{-7.3}$ to $10^{-8.0}$ and a standard deviation of 0.25. These ten titrations took place over a period during which the virus had been stored in a CO_2 box for 6 to 41 days. Even when sets of cultures were made up on different days using different batches of

embryo extract, plasma, and other constituents, the range of culture titers remained the same. The TC_{50} and ID_{50} of eight different batches of stock virus, titrated simultaneously in cultures and eggs, is shown in Table I. The range of the difference between TC_{50} and ID_{50} values

TABLE I

A Comparison of the TC_{50} and ID_{50} (Expressed as the Reciprocal of the Logarithm) of Eight Different Batches of Virus

Virus*	TC_{50}	ID_{50}	Difference
NDV p1	4.3	4.8	−0.5
NDV p18	5.5	5.3	+0.2
NDV p21	3.5	3.7	−0.2
NDV 5/12	8.3	8.7	−0.4
NDV 5/20	7.8	8.3	−0.5
NDV 7/29	8.3	8.7	−0.4
NDV 9/4	8.2	8.7	−0.5

* p1, p18, and p21 indicate that the virus used had been passed serially in chick heart tissue cultures 1, 18, and 21 times, respectively. The other samples are egg passaged stock virus obtained on different dates.

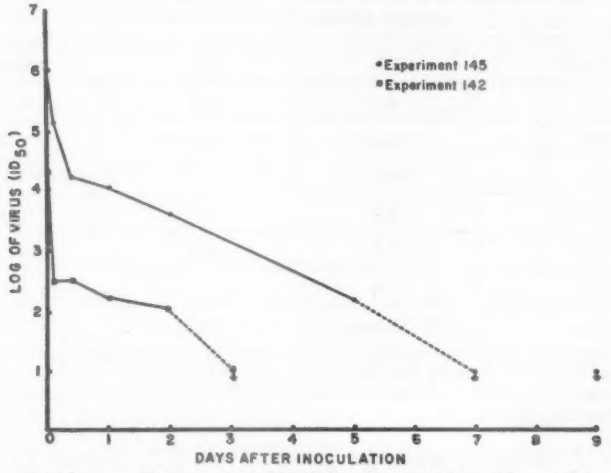
was $10^{-0.5}$ to $10^{+0.2}$, with the majority of TC_{50} values $10^{-0.4}$ and $10^{-0.5}$ lower than the ID_{50}.

The use of skeletal muscle fragments for titration of virus was compared to that of heart fragments. In three experiments the average TC_{50} value was $10^{-0.4}$ lower in skeletal muscle. Since this is within the range of experimental error for culture titration, skeletal muscle appears to be as satisfactory as heart for obtaining a titration end point. In another experiment, cultures of heart, lung, and liver were inoculated simultaneously with the same stock virus. The TC_{50} of the virus in heart tissue was $10^{-6.5}$, in lung $10^{-6.6}$, and in liver $10^{-6.4}$, indicating that outgrowths from all three tissues possessed similar susceptibility to the virus.

Survival of NDV in Medium Without Tissue. To comprehend the extent of active multiplication of the virus in roller tubes, the rate of virus degeneration in medium without tissue was determined. Stock virus was added to cell-free culture medium which had supported the growth of heart fragments for 2 days. The degeneration curve of the virus under these circumstances is illustrated in Text-figure 1.

There was a rather rapid initial decrease in the amount of virus which occurred within 3 to 7 hours after inoculation. The titer dropped steadily after this time.

Multiplication of NDV in Chick Embryonic Tissue. The growth of NDV in cultures was followed by determining the ID_{50} of pooled supernatants from four cultures. The extent of multiplication in the first hours after inoculation is shown partly in Text-figure 2. A significant increase of virus in the cultures which had received an in-

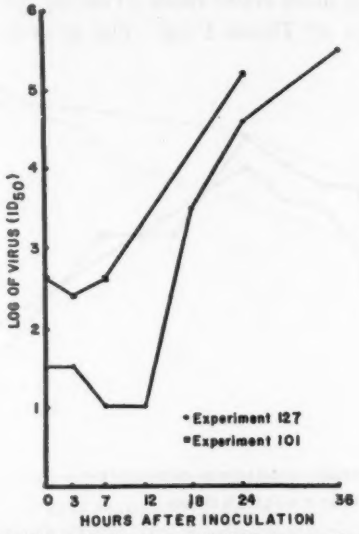


Text-figure 1. Degeneration of NDV in culture medium without cells.

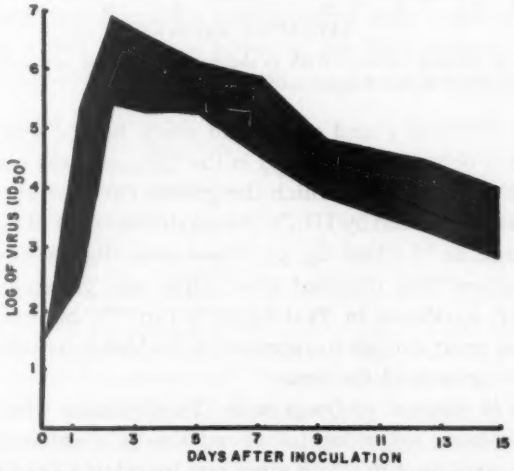
oculum of $10^{2.6}$ per ml. was first noted after 7 hours. In another experiment in which the inoculum was $10^{1.5}$ per ml., a significant increase was not noted until after 12 hours. Text-figure 3 illustrates the distribution of values from seven similar experiments in which one sample of stock virus was used and the inoculum in the tubes was $10^{1.5}$ per ml. The degree of ID_{50} variation in different experiments, as reflected by the shaded area, was expected, due to the errors inherent in the methods. The results show that maximum virus titers (near 10^{-6}) may be obtained from the tubes after 2 days, are fairly well maintained for 2 to 5 days, and gradually decrease, although a value of $10^{-3.0}$ may be expected after 15 days of incubation.

Growth curves using other batches of stock virus yielded results which fit into the same range of values as those shown in Text-figure 3. The maximum titer obtained in each case was never less than $10^{-5.2}$ and there was no significant decrease in the titer below this figure until 5 to 7 days of cultivation had elapsed. From that time, the curve was also the same as depicted in Text-figure 3. These considerations were important in attempting to analyze results when variations of

technique were used. It was interesting that in the majority of cultures maximum virus titers were obtainable a day or two before complete destruction of the tissue was noted.



Text-figure 2. Initial multiplication of NDV in heart roller tube cultures.

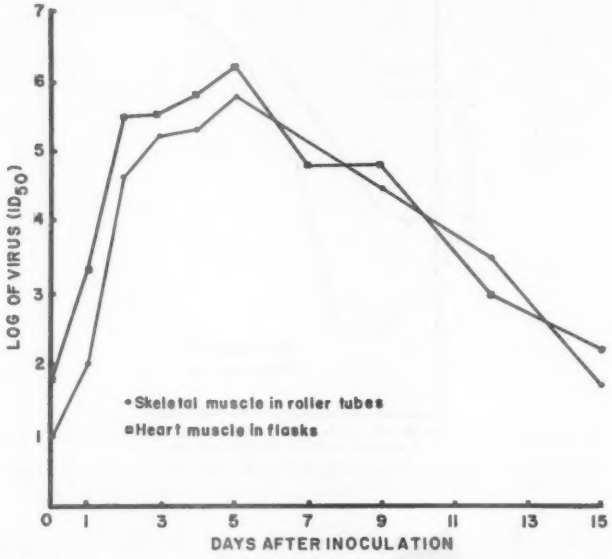


Text-figure 3. Growth curve of NDV in heart roller tube cultures.

Growth curves of virus in roller tube cultures of skeletal muscle and in 50 ml. Erlenmeyer flasks containing chick heart fragments sus-

pended in 4 ml. of liquid medium also were prepared. Examples are shown in Text-figure 4. When these techniques were used, maximum titers and growth patterns were obtained that did not differ greatly from those in chick heart roller tubes (Text-fig. 3).

Variation in Age of Tissue Used. The growth and tissue culture

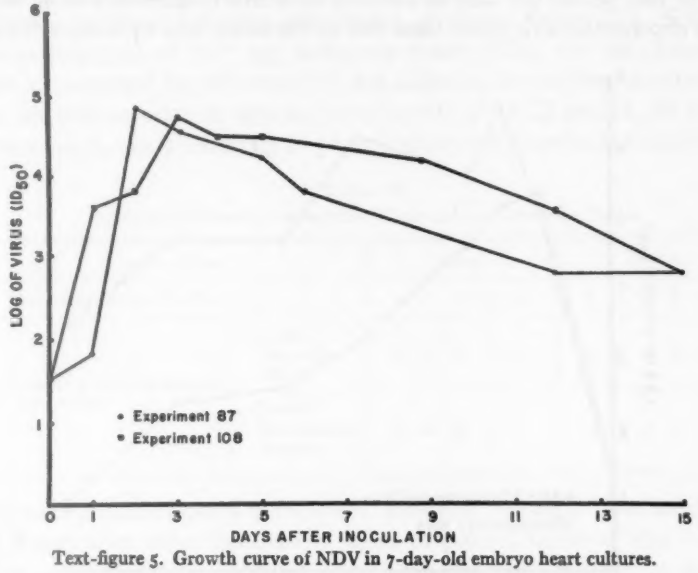


Text-figure 4. Growth curve of NDV in skeletal muscle roller tube cultures and heart fragments suspended in flasks of liquid medium.

titration of NDV in 7 and 19-day-old chick hearts were compared. No significant difference was noted in the TC_{50} of three sets of experiments. In the two cases in which the growth curve of virus in 7-day-old heart was established by ID_{50}'s, the maximum titers of virus reached were $10^{-4.8}$ and $10^{-4.7}$ (Text-fig. 5). These were slightly lower than the lowest maximum titer obtained when virus was grown in older embryonic heart as shown in Text-figure 3 ($10^{-5.2}$), but this difference in titer is not great enough to represent a decided inferiority of 7-day-old tissue for growth of the virus.

Variation in Amount of Outgrowth. To determine whether the degree of fibroblastic migration influenced the TC_{50} value of the virus, experiments were done in which virus was inoculated (1) immediately, (2) 2 to 3 days, and (3) 7 days after preparation of the cultures. Even though cultures were inoculated immediately, sufficient outgrowth

of cells occurred to permit evaluation of the TC_{50}. The TC_{50} values of these sets are shown in Table II.



Text-figure 5. Growth curve of NDV in 7-day-old embryo heart cultures.

The TC_{50} of the virus in sets inoculated immediately after preparation and before any migration had occurred were consistently lower than those in which virus was added at the later periods. However, regardless of the amount of outgrowth in cultures before inoculation,

TABLE II

TC_{50} of Virus in Sets of Cultures Inoculated Various Days after Preparation

Experiment no.	Day of inoculation		
	0	2–3	7
1	$10^{-6.8}$	$10^{-6.3}$	$10^{-6.7}$
2	$10^{-7.3}$	$10^{-6.8}$	$10^{-6.8}$
3	$10^{-6.7}$	$10^{-6.0}$	$10^{-7.7}$

TABLE III

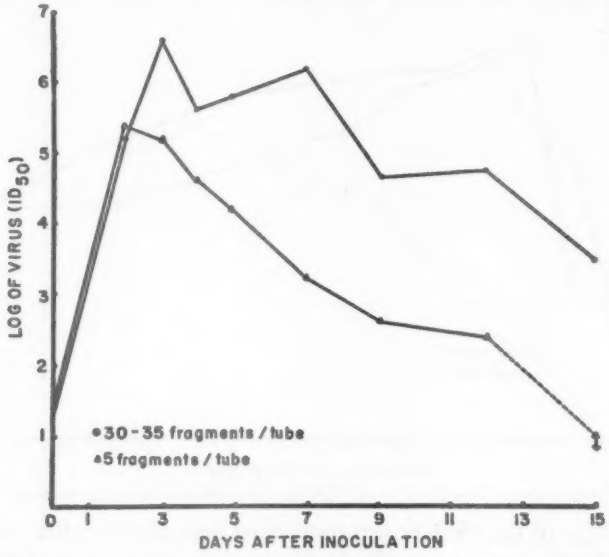
Comparative Infectivity of Passages 19, 20, and 21 for Eggs and Cultures (Expressed as the Reciprocal of the Logarithm)

Passage no.	ID_{50}	TC_{50}
19	5.0	5.3
20	4.6	4.8
21	3.7	3.5

the virus growth pattern and maximum titers reached in the supernatants of infected cultures were the same within estimated limits of error.

Influence of the Amount of Tissue in Cultures. When 5 instead of 30 to 35 fragments per tube were used as substrate for virus growth,

less virus was found in culture supernatants (Text-fig. 6). There was a similar initial increase in the titer of cultures at 24 or 48 hours. After this period the titer in cultures with five fragments was at least one exponential unit lower than that in the sister sets with more tissue.



Text-figure 6. A comparison of the growth curve of virus in cultures containing 30 to 35 fragments of heart with cultures containing 5 fragments.

Furthermore, in the sets with less tissue, there was an earlier drop in the titer as the cultures grew older.

Serial Passage of NDV. NDV was passed serially through 22 sets of chick heart cultures. This procedure had no effect on the infectivity of the virus for eggs. When simultaneous egg and culture titers were obtained from samples of serial passages number 19, 20, and 21, the values in Table III were obtained.

The ID_{50} and TC_{50} values indicate an equal degree of infectivity for both eggs and heart muscle cultures. In one experiment, virus which had been serially passed 19 times was titrated with both heart and skeletal muscle fibroblasts. The titers were $10^{-4.8}$ and $10^{-5.0}$ respectively, indicating no change in virulence for cells migrating from skeletal muscle due to serial passage through cultures of heart tissue.

Effect of Immune Serum. When 0.1 ml. of immune rabbit serum was added to embryonic heart cultures ½ hour before inoculation with 10-fold dilutions of virus, the TC_{50} was $10^{-2.5}$ compared to a value

of $10^{-6.8}$ obtained in cultures receiving 0.1 ml. of normal rabbit serum. The time required for adsorption and penetration of cells by the virus was investigated by adding 0.1 ml. of immune serum to cultures at various intervals following inoculation of virus (Table IV). With a virus inoculum of $10^{1.5}$ egg infectious doses (EID) per ml., immune serum protected the cells even if it was added 24 hours after inoculation. In another experiment with an inoculum of $10^{3.5}$ EID per ml., all tubes receiving immune serum up to 5 hours after virus inoculation contained

TABLE IV

Influence of Time on Prevention of Virus Action by Immune Serum

Virus inoculum		$10^{1.5}$/ml.					$10^{3.5}$/ml.				
Time after inoculation (hrs.)		1	3	5	8	24	1	3	5	8	24
Number of tubes destroyed per 4 tubes	0.1 ml. immune serum	0	0	0	0	0	0	0	0	3	4
	Control (no immune serum)	4	4	4	4	4	4	4	4	4	4

no microscopic evidence of infection. However, the addition of antibody 8 hours after virus inoculation failed to protect three of four tubes, and no tubes were protected when 24 hours were allowed to elapse between infection and addition of immune serum.

DISCUSSION

We have shown that it is possible to titrate NDV in cultures containing cells from various specific chicken embryo tissues. While this work was in progress, Fastier[2] reported on the titration of NDV in plasma clot and suspended cell cultures. He came to the conclusion that suspended fragments in liquid medium offered a better system for titration than plasma clots, since plasma interfered with good contact between cells and virus and lengthened the time necessary for total destruction to occur. In our experience, using a small quantity of plasma as the only material to support the cells, the time necessary to obtain the degree of destruction required to calculate a TC_{50} was 3 to 5 days after inoculation. Our technique was considered to be more expedient and satisfactory because of the thin plasma film, and because the tubes were not left stationary but rotated twelve times per hour. Although in cultures containing the highest dilutions of virus there were a few cells which were not destroyed after 5 days, the infection of these cultures was unmistakable. Eventually, all cells in such

cultures apparently were destroyed. The plasma clot technique, in this study, seems to be the more practical and is a dependable method for tissue culture titration of the virus.

The virulence of NDV for cells from various specific embryonic tissues was demonstrated. It was shown that our strain could cause destruction of cells migrating from brain, lung, liver, intestine, heart, and skeletal muscle. Topacio,[3] using hanging drop cultures, did not notice histologic changes of "skin, liver, intestines, etc." except for pyknotic changes in epithelial cells. Bang[4] noted a difference in the ability of three strains of NDV, which varied in virulence, to cause microscopic changes in epithelial cells from chorio-allantoic membranes. However, fibroblasts usually were destroyed. Thus, the cell predilections recorded by us might not hold true for all strains of NDV. It was noted that the TC_{50} obtained in skeletal muscle, heart muscle, liver, and lung were similar, indicating that these tissues were equally susceptible to infection by our strain. Embryonic chick heart was used in the routine titration of the virus because of the ease with which discrete fragments were obtained by chopping. If the less firm tissues were used, their handling and subsequent observation was not as easy.

If economy were desired, any of these tissues mixed together would probably prove a satisfactory substrate for use in titrations. From the results obtained by varying the techniques, 19-day-old hearts could have been used equally as well as 14-day-old hearts. It would also be possible to use less than 30 to 40 fragments of tissue per tube. However, to obtain the maximum TC_{50}, cultures should not be inoculated until after several days of migration, since we found that inoculation of freshly explanted fragments resulted in a lower value. The reason for this is obscure. It is possible that the exposed area of cells which have migrated in the form of a halo around the explants could offer an improved condition for adsorption of virus from the medium.

It was not anticipated to replace the egg technique for titration of NDV by a tissue culture method. It is conceivable that for certain experiments it might be helpful to use cultures of particular tissues for titration, in which case good reproducibility may be obtained by this method. In addition, the TC_{50} values obtainable in cultures approach the ID_{50} values in eggs.

The age of embryonic heart tissue used in cultures did not influence the TC_{50} value obtained in the cultures, but in two experiments using 7-day-old embryonic tissue, infected cultures did not yield as high an egg ID_{50} as that obtained with 14 or 19-day-old tissue; however, the

slight difference was of questionable significance. Nevertheless, for practical purposes, 7-day-old tissue was less satisfactory in these studies because of the large number of eggs required to obtain adequate tissue and because of the difficulty in obtaining discrete fragments for explantation.

When the growth curves of the virus are compared with the degeneration curve of NDV in medium without tissue, it is evident that most multiplication occurs between 7 and 48 hours after inoculation when inocula consist of $10^{1.5}$ to $10^{2.5}$ EID per ml. Equilibrium between growth and destruction is maintained for approximately 5 days, after which time more destruction than multiplication of virus takes place. The same pattern was followed regardless of whether heart or skeletal muscle was used, or whether heart fragments were suspended in a liquid medium. It is of interest that the maximum concentration of virus in cultures (ID_{50} of approximately 10^{-6}) was never as great as that found by egg inoculation.

The effect of NDV immune serum demonstrates the specificity of the virus action against the tissue. It is of interest that after the cultures had been exposed to the virus for an unusually long period, the addition of immune serum exerted a protective effect. Using an inoculum of $10^{3.5}$ infectious units per ml., it was shown that the immune serum prevented destruction if added within 8 hours after virus inoculation. With $10^{1.5}$ EID per ml., immune serum protected the cultures even if added 24 hours after virus inoculation. This appears to be contrary to *in vivo* experiments in which it was found that NDV was adsorbed within 5 minutes.[5] Two possible explanations may be offered: (1) physical conditions, e.g., interference by the plasma framework, were such that virus adsorption was delayed, or (2) there was an antibody excess sufficient to prevent spread of virus from cells initially infected by the inoculum.

Summary

The *in vitro* cultivation of Newcastle disease virus (NDV) in various chicken embryonic tissues and the destructive changes brought about by the virus have been described. NDV was titrated in roller tube cultures of heart fragments, the values being expressed as the "TC_{50}" of the virus. The reproducibility of the values which were obtained by this method were analyzed and compared with ID_{50} values from eggs. There was no significant difference between the two methods. Under the conditions of these experiments, an insignificant difference in TC_{50} occurred when skeletal muscle, lung, heart, and liver

tissue fragments were used, but a lower value was obtained when cultures were inoculated immediately after preparation, rather than allowing several days for outgrowth. Neither the age of embryonic tissue nor the presence of only five fragments in the tube affected the TC_{50} values.

The extent of multiplication of NDV in cultures of 7 and 19-day-old embryonic heart was approximately the same; neither did the amount of outgrowth which was allowed to occur before inoculation influence the maximum titer of virus in supernatants. When 5 instead of 30 to 35 fragments were used, the growth curve of the virus showed an earlier decrease of the titer.

Immune serum was shown to inhibit destruction of tissue by the virus, even when it was added after an unusually long exposure of the tissue to the virus.

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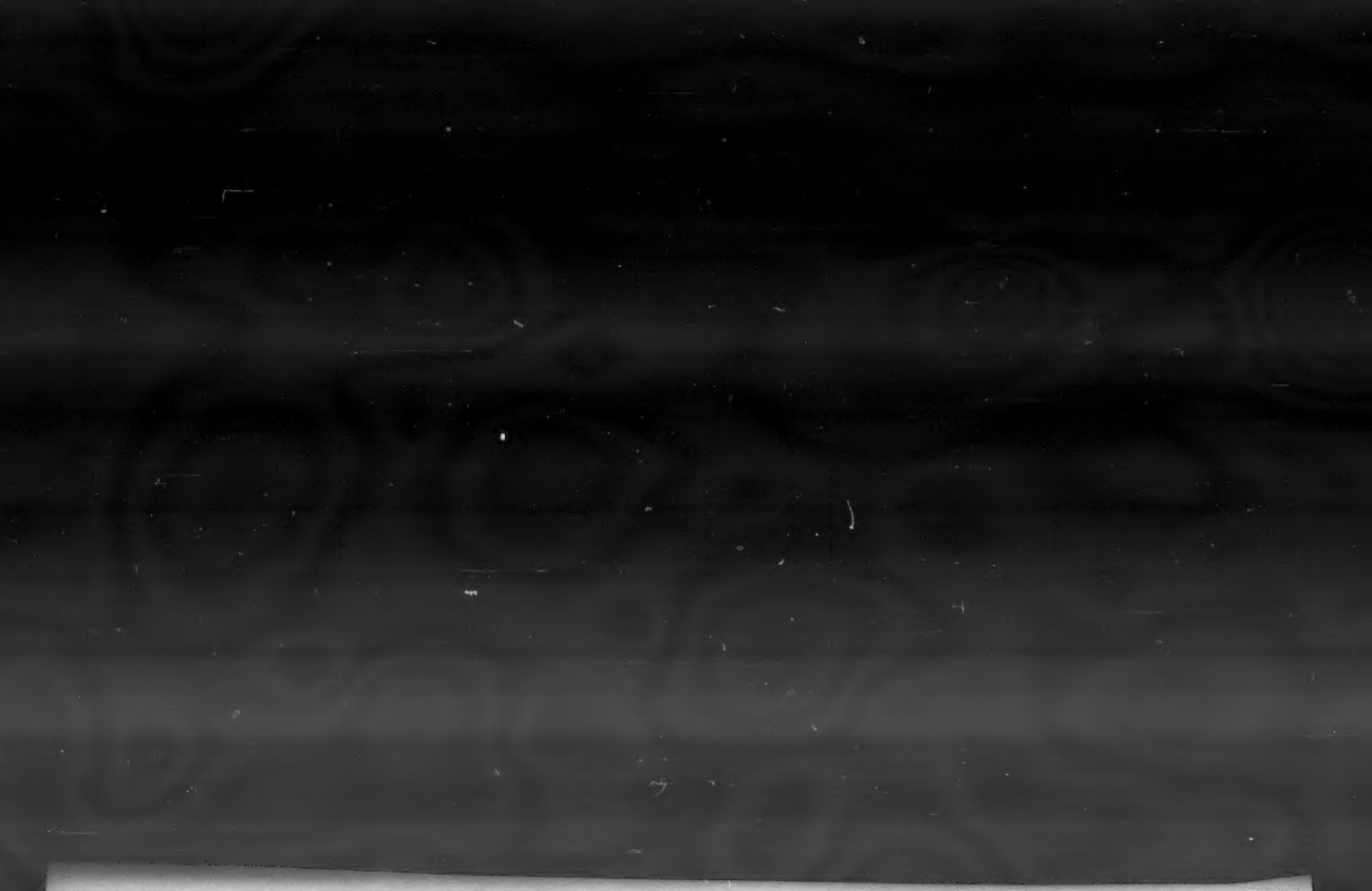
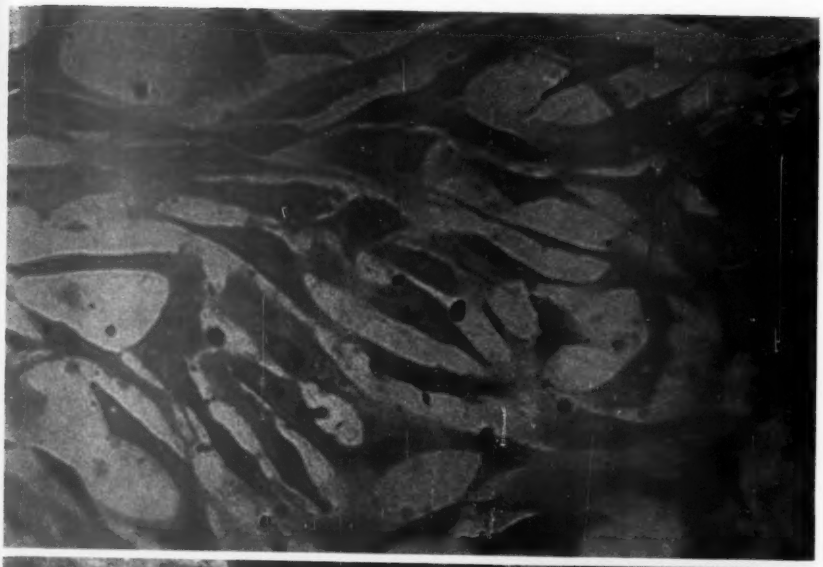
LEGENDS FOR FIGURES

FIGS. 1 to 4. Fibroblasts from chick embryo heart. Giemsa's stain. × 264. *Fig. 1.* Fibroblasts from an uninoculated control culture. *Fig. 2.* "Beginning destruction" in a culture inoculated with NDV. Rounding and clumping of cells near the explant may be noted. *Fig. 3.* "Partial destruction" by virus, showing clumping, vacuolization, and granularity of cells. *Fig. 4.* "Complete destruction" of fibroblasts. Clumps of cell débris and strands of degenerated cytoplasm are shown.

FIGS. 5 and 6. Chick embryo intestine. Giemsa's stain. × 264. *Fig. 5.* Intestinal epithelial cells from an uninoculated control culture. *Fig. 6.* "Complete destruction" of cells by virus.





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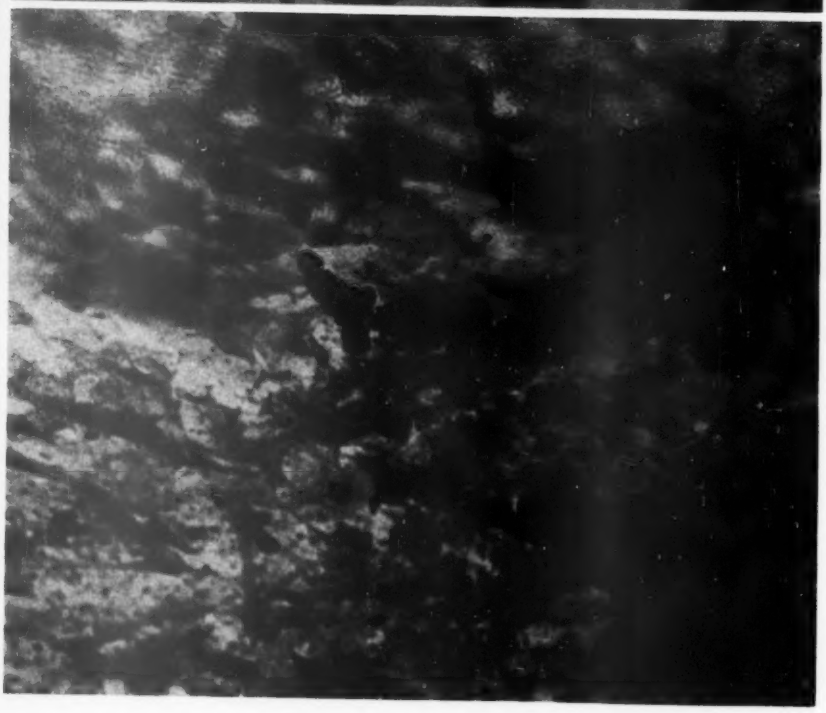

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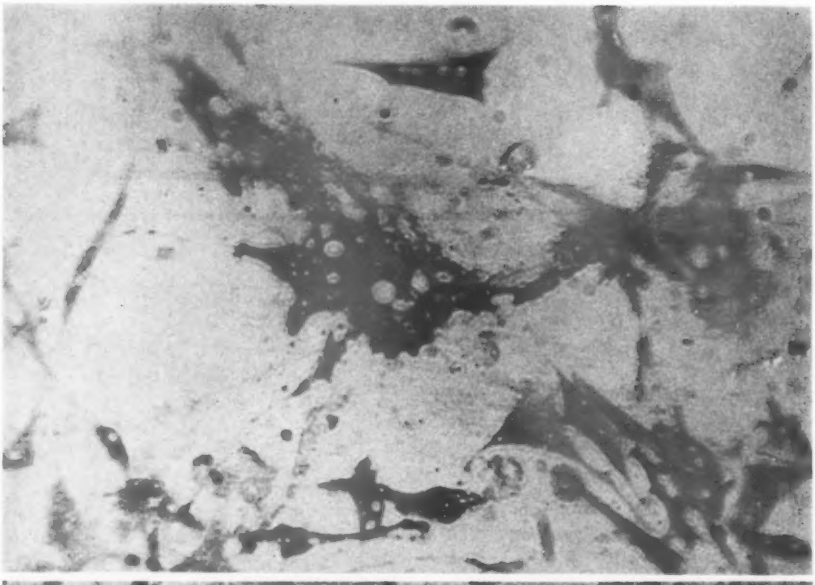

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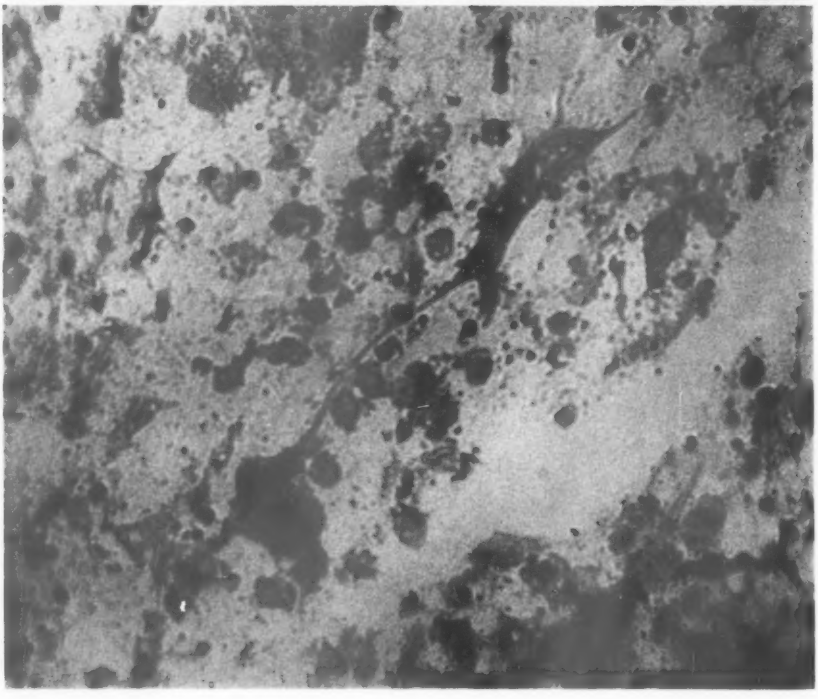

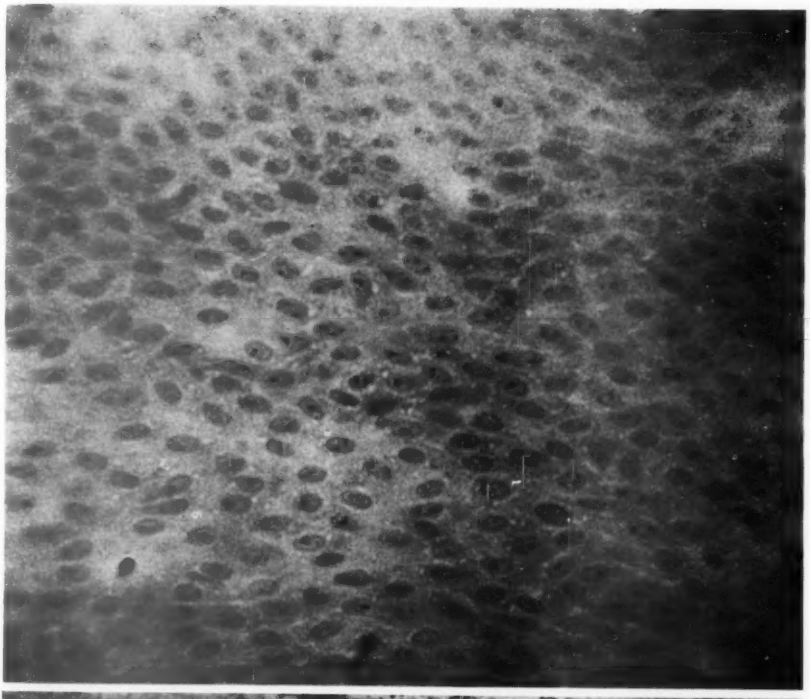

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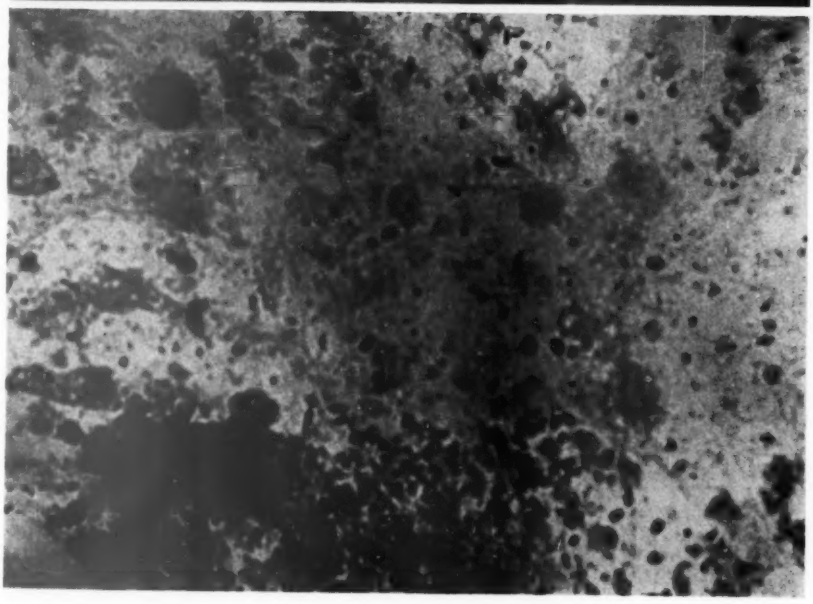

6

PATHOLOGY OF POLIOMYELITIS IN THE CHICK EMBRYO*

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Three years ago the MEF1 (Lansing) strain of poliomyelitis was successfully adapted to the developing chick embryo by Roca-Garcia, Cabasso, *et al.*[1,2] These authors did not observe any gross lesions in the infected embryos. The present study was undertaken to determine the nature of poliomyelitis in the chick embryo, in the hope of extending our knowledge of the pathogenesis and manifestations of the disease.

MATERIAL AND METHODS

Virus Inoculum. The virus used was derived from the egg-adapted MEF1, type 2 (Lansing) strain of poliomyelitis virus, series R 302 and R 382.[1] A new series, R 302 + 382, was established in the chick embryo with a suspension prepared from the brains of embryos of the serial egg passages R 302-33 and R 382-30 on the 14th day after inoculation. The inoculum for the present study was prepared from the 3rd egg passage of the new series. It consisted of 0.4 ml. of a 20 per cent chick embryo suspension in buffered saline solution, and contained 5,330 mouse LD_{50} doses of virus.

Control Inoculum. A second group of embryos was inoculated with 0.4 ml. of a 20 per cent normal chick embryo suspension to serve as controls.

Embryos. Two groups comprising 201 and 54 7-day-old White Leghorn chick embryos were inoculated with the virus and the control inocula respectively, and were incubated at 36° C. Embryos were candled daily and the mortality noted (Table I). Live embryos were sacrificed as shown in Table I for a study of the morphologic features and for estimation of the virus content of the tissues.

Estimation of Virus Content of Embryo Material. Blood, yolk sacs, brains, and embryo bodies were harvested separately. Yolk sacs were drained before use, brains were obtained by dissection, and blood was collected from the body after decapitation. This manner of collection resulted in some dilution of the blood by amniotic and allantoic fluids, but previous experiments have shown that only small traces of virus are present in these fluids.[1] The yolk sacs, brains, and embryo bodies were prepared as 10 per cent suspensions in buffered saline solutions

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by means of Waring blendors or Ten Broeck grinders. Suspensions were centrifuged at 1,500 r.p.m. for 10 minutes. Serial tenfold dilutions of the supernatant and of the blood were inoculated into Swiss mice weighing 10 to 12 gm. The mice were observed for 21 days, and the LD_{50} titers were calculated by the method of Reed and Muench.[3] The LD_{50} titer of the original virus inoculum (pooled embryonic tissues) was estimated in the same manner.

Morphologic Examination. After removal of the membranes, the embryos were weighed and examined macroscopically. The material

TABLE I

Disposal and Fate of Control and Virus-Infected Chick Embryos

Day after inoculation	Mortality				Morphologic examination		Determination of virus content
	Control		Virus infected				
	No. dead	Per cent*	No. dead	Per cent*	Control	Virus infected	
1	4	7	18	9	3	3	6
2	9	18	5	3	3	3	3
3	0	0	1	1	3	3	3
4	0	0	9	6	3	3	3
5	0	0	8	6	3	3	3
6	0	0	8	6	3	3	3
7	1	4	4	4	3	3	3
8	0	0	1	1	3	3	3
9	0	0	1	1	3	3	2
10	0	0	0	0	3	3	2
11	0	0	0	0	3	3	2
12	0	0	4	5	3	3	2
13	1	25	5	7	2	3	2
14	0	0	7	11	1	3	2
15†	–	–	10	20	–	3	2
16‡	–	–	18	55	–	2	1
17	–	–	2	40	–	1	1
18	–	–	0	0	–	1	–

– = No results available because all embryos were used up.
* Per cent of embryos under observation on that day.
† One hatched.
‡ Seven hatched.

was then fixed in Bouin's solution, embedded in paraffin, and 6 μ sections were cut.

Whenever possible, 3 embryos were sacrificed each day from the control and from the virus-infected groups (Table I). Two of the 3 from each group were bisected in a parasagittal plane slightly to the left of the midline. Serial sagittal sections were cut through the right

portion of one; the right portion of the other was kept in reserve for use when parts of the first were lost or damaged. Blocks were prepared from the 3rd embryo in each group by coronal sections in the following planes: (a) base of the beak, (b) middle of the eyes, (c) behind the eyes, (d) external auditory meatus, (e) midway between the ear and the neck, (f) neck, (g) upper and (h) middle thorax, (i) middle and (j) lower abdomen. Since no lesions were observed in the sagittal sections of the forebrain, serial sections were limited to the brain stem and to appropriate blocks when the ciliary ganglion or the posterior nerve root ganglia were absent in the first 3 slides from each block. In addition, sections of limbs (longitudinal and transverse), chorio-allantoic membrane, and yolk sac also were prepared from all the embryos which were sectioned. In order to achieve the most complete examination and at the same time to allow spare sections for special stains, the following method was adopted. After trimming the block, 3 slides were prepared by mounting the first 3 to 12 sections, depending on the size of the embryo. When serial sections were made, the next 9 to 29 sections were discarded, 3 slides were prepared as before, and the procedure was repeated until the block was used up. The first of the 3 slides in each group was stained with hematoxylin and eosin, and the remainder were kept for special stains.

For the day before the onset of recognizable degeneration in hematoxylin and eosin preparations, and when lesions were doubtful or unexpectedly absent, the spare sections were stained with buffered thionine (pH 6.2 to 5.0) or with Barrett's stain.[4] The latter proved especially useful in the earlier embryos in which the poorly developed Nissl substance stained with the blue mixture and the degenerating nerve cells took the red stain. In addition to thionine and Barrett's stain, special stains were applied to sections of the central nervous system, cranial and posterior nerve root, and autonomic ganglia from the time when embryos showed the earliest lesions until the end of the experiment; and to the same tissues from uninfected embryos of the same ages, as follows: Holmes's silver method for neurofibrils,[5] Mann's eosin methyl blue, Sudan black B, and the Loyez method for myelin.

Sections containing abnormal skeletal muscle and myocardium also were stained by Mallory's aniline blue, Lendrum's reticulin,[6] and Heidenhain's iron hematoxylin methods and with Giemsa's stain.

Results

Mortality. In both the uninfected and virus-infected groups some embryos were found dead during the first 2 days after inoculation

(Table I). Since the percentage mortality was slightly higher in the uninfected group, the deaths could not be attributed to the action of the virus, and were almost certainly the result of trauma during inoculation. The high mortality in the virus-infected group after the 14th day was probably caused by unfavorable hatching conditions. Comparable statistics for the controls were not available, since all the embryos in that series were used up before the time of hatching. From the 3rd to the 14th day after inoculation of the eggs, except for 2 uninfected embryos which died, there was a consistently higher percentage mortality in the virus-infected group (Table I).

TABLE II

Weights of Chick Embryos (Mean of 3 Embryos)

Day	Control	Infected
	gm.	gm.
8	1.10	1.60
9	1.67	1.67
10	1.93	2.60
11	2.40	2.77
12	3.97	4.33
13	5.03	5.10
14	7.23	8.73
15	8.97	10.37
16	11.83	11.27
17	15.83	14.90
18	19.33	17.77
19	20.67	17.23
20	21.85	21.33
21	26.20	24.40

Growth. Statistical analysis of the weights of the infected and uninfected embryos (Table II) reveals that the difference in weights (infected-control) could be represented by a 2nd degree equation:

$$d = +0.16 + 0.21x - 0.028x^2$$

where d is the difference and x is the number of days after inoculation of virus. This curve is shown in Text-figure 1. From the 2nd to the 6th day the infected group was about 0.5 gm. heavier than the controls; the difference then grew smaller, reaching 0 on the 8th day (Text-fig. 1). After the 10th day the infected group was significantly lighter.* Therefore, poliomyelitis infection was responsible for an inhibition of growth in the later stages of embryonic life.

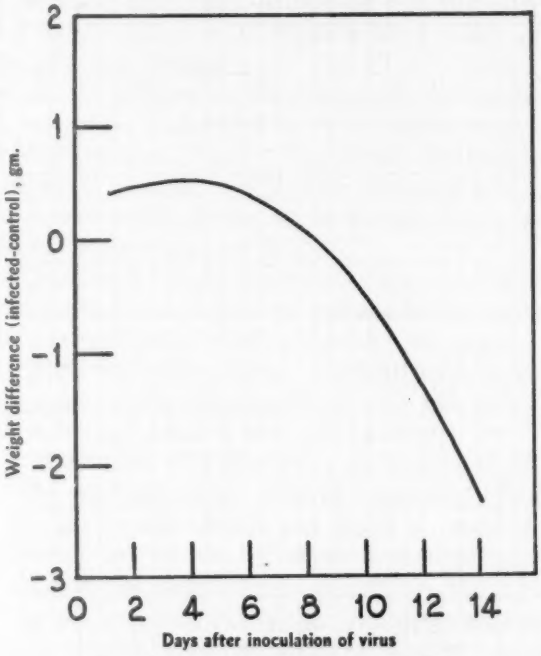
MORPHOLOGIC FINDINGS

Macroscopic

No deformities or other pathologic changes were observed in the great majority of embryos. Striking congenital abnormalities were present in 2 virus-infected embryos (no. 1, and no. 9 of Table III). In the first, an 8-day-old embryo, agenesis of the left eye with failure of development of the lens gave the appearance of a one-eyed monster. The other abnormality comprised a partial dicephalus in a 12-day-old embryo (Fig. 1). There were 2 pairs of optic nerves, 2 beaks and nasal cavities, 2 sets of jaw bones, and 2 imperfectly developed

* The test of significance was done at the 5 per cent probability level.

brains with lateral ventricles and a number of channels which appeared to represent parts of the 3rd ventricles. Partial fusion occurred in the region of the optic lobes, and was complete below the level of the cerebellum. Although there was complete duplication of the eyes, the median pair were contiguous and were served by one orbit and one set



Text-figure 1. Daily differences in weight.

of eyelids (Fig. 1). In this same embryo a defective enclosure of the primitive neural tube by mesoderm resulted in a meningocele over the optic lobes.

Microscopic

A clear differentiation of the nuclei of the brain was not possible before the 10th day of incubation. The identification and nomenclature of the nuclei were based on the description of the robin brain by Papez[7] and on the material collected by Kappers *et al.*[8] Accurate separation of the 9th and 10th and the 11th and 12th cranial nerve nuclei could not be made. In the absence of any separating grooves or localized grouping of neurons, it was not possible to differentiate the corpora quadrigemina.

TABLE 3 DISTRIBUTION AND SEVERITY OF SPECIFIC LESIONS OF THE NERVOUS SYSTEM IN CHICK EMBRYOS AFTER INFECTION WITH POLIOMYELITIS VIRUS ©

NO.	DAY OF INCUBATION	CRANIAL NERVE GANGLIA *						POSTERIOR NERVE ROOT GANGLIA				CRANIAL NERVE NUCLEI									SUBSTANTIA RETICULARIS OF PONS & MEDULLA	CORPORA QUADRIGEMINA	CEREBELLAR ROOF NUCLEI	SPINAL CORD (ANTERIOR HORN)			
		V	VII	VIII	IX PETROSAL	X JUGULAR	X NODOSUM	CERVICAL	BRACHIAL	THORACIC	LUMBAR	III	IV	V MOTOR	VI	VII	VIII COCHLEAR	VIII VESTIBULAR	X	XII				CERVICAL	BRACHIAL	THORACIC	LUMBAR
5*	10	+++	+	0	0	0	0	-	-	-	-	-	-	++	-	++	-	+	0	0	+	+	-	-	-	-	-
9*	12	+++	++	-	+	++	++	++	+++	+	++	++	-	+	-	++	-	0	0	0	++	0	-	+	++	+	++
10		++++	+	++	0	0	+++	+++	+++	+++	+++	-	-	++	+	++	+	++	0	0	+++	+	++	+	++	++	++
11*	13	+++	+	+	0	0	0	++	-	++	+	-	-	+	+	+	-	+	0	0	+	-	++	+	-	+	-
12		++++	+	+	+	+	++	+++	+++	+++	+++	+	+	+	-	+	+	+	++	-	+	+	+	+	+	+	++	++
13*	14	++++	++	+	0	0	+	++	+++	++	++	-	-	+	++	++	0	+	0	0	+	+	-	+	+	+	+
14		++	++	+	-	-	-	++	+++	++	+++	-	-	++	+	+	+	+	-	+	++	-	+	+	+	+	++
15*	15	+++	-	-	-	++	0	++	+++	++	+++	-	0	+	-	++	-	+	+	-	+	-	-	+	+	+	++
16		+++	+	-	++	+	+	++	+++	+++	+++	+	-	+	-	++	-	+	+	-	+	+	-	+	++	++	++
17*	16	+++	-	+	-	+	0	+	+++	++	+	-	-	-	-	+	-	-	-	-	+	-	-	+	+	++	++
19*	17	++	+	-	-	-	0	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
20		+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21*	18	+++	++	+	+	+	0	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
22		+++	+	-	-	+	+	+	++	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
23*	19	++	0	-	+	+	0	+	+	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
24		++	-	+	-	+	+	+	++	+	++	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+
25*	20	+	0	-	0	++	0	+	-	-	-	-	-	-	0	0	-	-	-	-	-	-	-	-	-	-	-
26		+++	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
27*	21	++	+	+	+	++	0	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
28		++	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
29*	22	+	+	-	0	0	0	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30		+	+	-	-	-	0	+	+	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
31		+	-	-	0	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32*	23	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
33		+	+	-	-	+	-	-	-	-	-	0	-	-	-	-	-	-	-	-	+	-	-	-	-	-	0
34	24	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
TOTAL NO. OF LESIONS		26	16	10	7	17	8	22	19	18	20	3	1	9	4	12	3	8	4	1	14	9	4	10	10	11	12
PERCENTAGE OF STRUCTURES WITH LESIONS		100	67	40	37	81	53	85	73	72	80	12	4	35	16	48	12	32	19	5	54	36	15	38	38	44	50

* = EMBRYOS SECTIONED IN THE CORONAL PLANE; THE OTHERS WERE CUT SAGITTALLY.

\+ = TWO OR THREE NEURONES DAMAGED.

++++ = MORE THAN TWO-THIRDS OF NEURONES DAMAGED.

++ and +++ = INTERMEDIATE DEGREES OF DAMAGE.

© = EMBRYOS SHOWING NO SPECIFIC LESIONS ARE NOT INCLUDED. THEIR NUMBERS AND DAY OF HARVEST ARE AS FOLLOWS: NOS. 1 & 2, DAY 8; 3 & 4, DAY 9; 6, DAY 10; 7 & 8, DAY 11; 18, DAY 16; 35 DAY 25

\- = STRUCTURE EXAMINED BUT NO LESION SEEN.

0 = STRUCTURE NOT EXAMINED.

ß = NO SPECIFIC LESIONS IN CILIARY GANGLION.

Non-Specific Lesions

Non-specific lesions were of three kinds: (a) definite abnormalities which occurred in the virus-infected group, but which have been described by others in normal embryos[9]; (b) deviations from the accepted normal histology of the adult chick which occurred with equal frequency in the infected and uninfected embryos; (c) deviations which were more common or more pronounced in the virus-infected than in the uninfected group.

Category a. In addition to the congenital malformations already described, category *a* included a focus of hepatic necrosis in a 12-day-old infected embryo (Fig. 2). The process was unassociated with any inflammatory reaction; a thin layer of intact cells beneath the capsule suggested that the lesion was of vascular origin, and the subcapsular blood supply was intact (Fig. 2).

Category b. Recent minute hemorrhages were fairly common in all parts of the mesenchyme of infected and uninfected embryos in category *b*. In 3 virus-infected embryos (nos. 24, 27, and 30, Table III) there were small foci of less recent cerebral hemorrhage associated with phagocytosis of degenerating blood cells and hemosiderin, and necrosis of occasional neuronal and glial elements.

Throughout a period of 5 days from the time when the tangential nucleus of the vestibular nerve could be recognized (10-day-old embryo), a peculiar hyaline change was found in many of its bipolar neurons. Except for the vestige of an occasional nucleus, earlier stages of neuronal degeneration were absent, and the typical appearance was that of numerous completely homogeneous, faintly eosinophilic, bipolar cells in which no trace of Nissl substance could be demonstrated (Fig 3). The phenomenon was equally common in control and in infected embryos, and was unaccompanied by any inflammatory reaction.

Changes of a degenerative nature were observed also in the larger neurons of the ciliary ganglion from the 12th day onward. The pattern of the Nissl substance in many of these cells was that of peripheral or central chromatolysis of varying degree (Fig. 4); in a few cells complete chromatolysis occurred (Fig. 4) and destruction was completed by karyolysis, shrinkage, and solution of the cell.

Throughout embryonic life the Nissl substance of most of the neurons of the posterior nerve roots and analogous cranial nerve ganglia was distributed around the periphery of the cell, simulating central chromatolysis (Fig. 5). A similar, but less marked, peripheral distribution of the Nissl substance was common in the

neurons of the anterior horns, nucleus of Hoffmann,[8] formatio reticularis of the medulla, and the motor cranial nerve nuclei of the younger embryos; the Nissl substance increased as the embryo developed, and by the 18th to the 20th day of incubation it filled the cytoplasm. There was no other evidence of degeneration of the affected cells.

After the 19th day of incubation, peripheral vacuolation of occasional neurons of the posterior nerve root ganglia was observed, and the Nissl substance of the cell was aggregated around the nucleus. Transitional stages between change of this type and liquefaction of the affected neuron sometimes were seen.

Category c. A form of degeneration of the skeletal muscle (category *c*) occurred in 57 per cent of the infected, and 41 per cent of the uninfected embryos. This myopathy was observed in one infected embryo as early as the 9th day of incubation, and the most extensive degeneration was found in a 16-day infected embryo; no lesions were present in the nervous system of either of these embryos. On the whole, the muscle changes were most conspicuous in the older embryos and were present in 12 of the 13 infected embryos harvested after the 18th day of incubation.

The process involved individual fibers and, in particular, those of larger caliber in the posterior occipital group and in the leg. The earliest change consisted of swelling, loss of the cross striation, increased eosinophilia, and fusion of the myofibrils in a segment of the affected fiber. The more advanced stages of degeneration were characterized by pyknosis of the muscle nuclei, fragmentation of the sarcoplasm and, finally, absorption of the débris (Fig. 6). The sarcolemma remained intact after the fiber had been absorbed (Fig. 6). These changes were accompanied by some edema of the interstitial tissue but, with the exception of 2 embryos which failed to hatch (nos. 33 and 34, Table III), there was no inflammatory cellular reaction. In these 2 birds there was considerable perivenous and diffuse infiltration of the affected muscle by macrophages, mast cells, lymphocytes, and heterophilic myelocytes and polymorphonuclear leukocytes. Some of the disintegrating fibers had been invaded by macrophages and were undergoing phagocytosis (Fig. 7). In preparations stained with Giemsa's stain, no organisms could be seen.

In order to distinguish between physiologic and pathologic accumulations of myeloid cells, a careful study of the distribution of these cells was made. Extramedullary myelopoiesis was observed in the mesenchyme of all embryos. Occasional myeloid cells were present in the developing meninges and around the blood vessels in the inter-

vertebral foramina and the angle of the orbit as early as the 8th day of incubation. Myelopoiesis in the mesenchyme increased with advancing age and reached a maximum in amount and extent between the 12th and 19th days of incubation. Foci of developing myeloid cells usually were found near blood vessels and were particularly common beneath the lateral aspect of the base of the cranium, in the roof of the mouth and upper beak, orbit, posterolateral cervical subcutaneous tissue, meninges (especially around the anterior spinal artery), intervertebral foramina near the root ganglia, pancreas, bursa of Fabricius, proventriculus, ceca, spleen, jugular and pelvic lymph sacs, liver, and yolk sac. A few myeloid cells were seen occasionally near the blood vessels of the myocardium. After the 20th day of incubation, myelopoiesis gave place to lymphopoiesis in the bursa of Fabricius. The physiologic atrophy of the yolk sac was accompanied by a diminution in hemopoiesis and the disappearance of all primitive cells by the 19th day of incubation. Medullary myelopoiesis became increasingly active from the 10th day on.

In the infected and uninfected embryos there was no significant difference in the amount and distribution of myeloid cells except in the meninges, around the posterior nerve root ganglia and, of course, in relation to definite lesions as described elsewhere. Myeloid cells were slightly more numerous in the meninges, and especially around the anterior spinal artery and intervertebral vessels in all the infected embryos showing specific lesions.

Lesions Confined to Virus-infected Embryos

Neural Lesions: Course and Distribution. The incidence, distribution, and an indication of the severity of neuronal degeneration are presented in Table III. During the first 4 days after inoculation of virus, lesions were found in one embryo only (no. 5, Table III). After this period, pathologic changes were present in all but 2 embryos (nos. 18 and 35, which were harvested on the 16th and 25th days of incubation, respectively). The acute phase of the disease, when the changes were most widespread and most intense, occurred between the 12th and 16th days of incubation (i.e., between the 5th and 9th days after inoculation of virus). Throughout the remaining 9 days of observation, degenerating cells within the central nervous system were few and irregular in distribution, and the lesions in the ganglia became progressively less extensive and less common (Table III).

In the first embryo, which showed lesions on the 10th day (no. 5, Table III), the spinal cord and ganglia were intact. During the acute

stage of the disease, lesions were widespread throughout the posterior nerve roots and cranial nerve ganglia, the anterior horns at all levels of the spinal cord, the substantia reticularis of the medulla and pons, the motor cranial nerve nuclei and the vestibular nucleus, roof nuclei of the cerebellum, and the corpora quadrigemina (Table III). Neuronal degeneration was most constant and most severe in the 5th and 7th cranial nerve ganglia and motor nuclei, posterior nerve root ganglia and spinal anterior horn cells, the vestibular nucleus, and the substantia reticularis of the brain stem (Figs. 8 to 16). The peripheral neurons of the ganglia were less severely affected than those in the center (Figs. 8 and 15). The ciliary ganglia were not significantly different from those of the uninfected controls (Fig. 4.) When the anterior horns were severely damaged, occasional neurons around the central canal and in the posterior horns also were destroyed. The nucleus of Hoffmann was unaffected. In addition to the lesions described in Table III, some neuronal degeneration was observed in the semilunar nucleus (nos. 5, 10, 11, 12, and 13), and less commonly in the superior and inferior olives, the intermediate nucleus of the lower medulla, the deeper layers of the optic tectum (stratum griseum centrale), the nucleus isthmi, and the lateral mesencephalic nucleus. Degeneration of a rare cell in the sympathetic ganglia was seen also, but despite the presence of severe lesions in the adjacent posterior nerve root ganglia the sympathetic neurons usually were unaffected (Figs. 9 and 10).

After the acute phase, degenerating nerve cells were found mainly in the ganglia, where they persisted until the 24th day of incubation. No lesions were present in the one embryo examined on the 25th day. The most striking and persistent neuronal degeneration was seen in the trigeminal ganglion (Fig. 8), which was affected in every embryo showing lesions (Table III).

Nature of the Lesions. The course of neuronal destruction appeared to be of variable, but mainly of short duration, since all degrees of degeneration, including complete lysis of the cell, were observed in the earliest embryos showing lesions (Fig. 10). In particular, the first stages of destruction must have been rapid, since cells showing early changes were scanty throughout. They were present, however, from the 3rd to the 7th day after inoculation of virus, so that the initiation of the destructive process must have continued during this period. After the 7th day, many of the degenerated neurons had been absorbed and all the remaining abnormal cells showed advanced degenerative changes and no evidence of regeneration.

As in mammals,[10,11] the first abnormality was a reduction in size and staining capacity of the Nissl bodies throughout the cytoplasm (Fig. 11). This resulted in an accentuation of the pre-existing appearance of central chromatolysis (cf. Figs. 5 and 11). As chromatolysis progressed, the cytoplasm of the affected cell became swollen (Fig. 11), and degeneration continued in two ways. One form, which appeared to be a hyperacute process, occasionally was observed in the anterior horn (Fig. 12) and less commonly in the ganglia (Fig. 10) as early as 3 days after inoculation of virus (no. 5, Table III). The cell continued to swell, the Nissl substance completely disappeared, the nucleus underwent rapid karyolysis without any previous structural change, and the cell was completely destroyed by peripheral and finally central vacuolation (Fig. 12). More commonly, however, the course of cellular destruction was less rapid. The nucleus was displaced to one side and sometimes indented to form a semilunar structure (Figs. 11 and 13). Uneven nuclear shrinkage followed, and irregular margination of the chromatin was common. The nucleolus often was fragmented and displaced to one side, and in some cells clumping of the parachromatin produced the characteristic acidophil "inclusion bodies" described by Covell[12] and Hurst[13] (Fig. 13). Meanwhile, karyolysis proceeded at a variable rate, and at any point in the series of changes described, the chromatin appeared to undergo solution and disappear from the cell (Fig. 11). Finally the cell remnant shrank, and a faintly stained shadow was left (Figs. 8 to 11). In the central nervous system absorption was fairly rapid, and only a very few "shadow" cells were found after the 16th day of incubation (Table III). The final dissolution of the cell was slower in the ganglia, where shrunken remnants of cells in the center of signet rings, formed by the surviving amphicytes, persisted in larger numbers up to the 24th day (Table III).

The neurofibrils showed no changes until chromatolysis was complete and nuclear degeneration far advanced (Fig. 14). At this stage, some of the fibrils became thin and lost their affinity for stain (Fig. 15) and others appeared irregularly thickened and broken (Fig. 14). Similar changes were observed in the axons of the anterior and posterior nerve roots from the 6th to 12th days after inoculation of virus. The Loyez preparations were disappointing, and no demyelination could be detected by the Sudan black method.

Apart from the presence of occasional, round, primitive, mononuclear cells in the lumen, or more rarely in or near the wall of a congested blood vessel, neuronal degeneration and absorption within the central nervous system proceeded without any inflammatory cellular

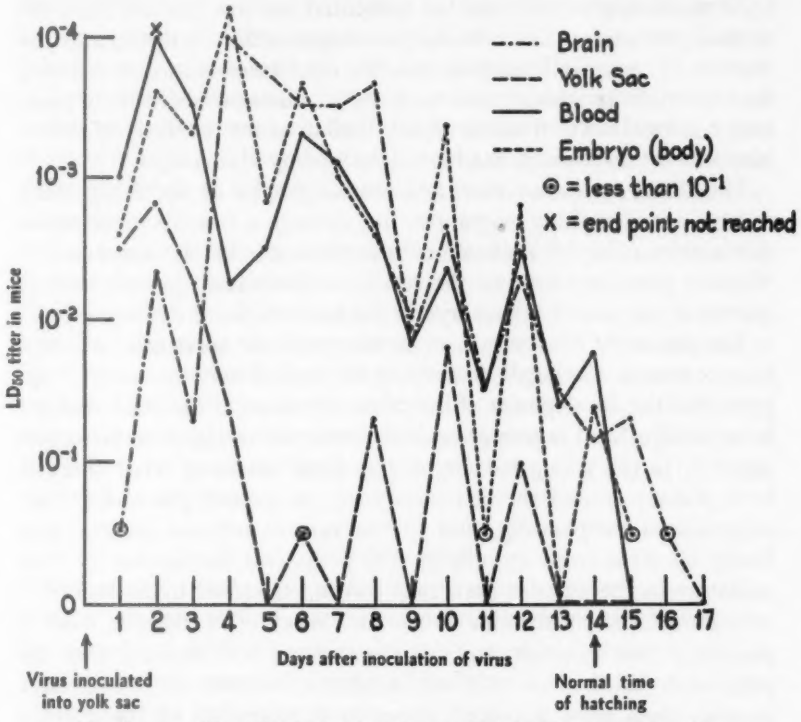
reaction (Figs. 12 and 13). No evidence of multiplication or degeneration of amphicytes was detected (Figs. 8 to 11). In a few embryos after the 17th day of incubation, one or at the most two small inflammatory foci were seen. In 5 of these embryos abnormal venules were found in the vicinity of degenerating neurons in the substantia reticularis of the medulla or the anterior horns. Pleomorphic mononuclear cells and lymphocytes were present in the wall, Virchow-Robin space, and to a lesser degree in the surrounding tissue. Neuronophagy by the mononuclear cells was rare. A small focus consisting entirely of pleomorphic mononuclear cells (probably microglia) was observed in the optic tectum of one embryo (no. 25) and in the thalamus of another (no. 31).

The extensive degeneration of ganglionic neurons on the 3rd, 5th, and 6th days after inoculation of virus was accompanied by a slight increase in the number of perivascular myeloid cells around the periphery of the ganglia, and, less commonly, by minimal perivascular myeloid infiltration within the ganglia (Fig. 16). During the next 2 days there was considerable perivascular and diffuse infiltration of the affected ganglia by myeloid cells and mononuclear cells resembling large lymphocytes (Fig. 8). The inflammatory reaction subsided completely during the next 3 days, except for the persistence of a slight increase in the number of perivascular myeloid cells in the periganglionic mesenchyme.

Extraneural Lesions. Myocarditis was observed in 6 embryos (nos. 23, 28, 29, 30, 31, and 33, Table III). In the 5 older embryos there were ill defined areas in all parts of the myocardium of infiltration of the interstitial tissue by mononuclear cells, lymphocytes, and occasional heterophilic polymorphonuclear leukocytes (Fig. 17). In the more densely infiltrated zones, the inflammatory reaction was accompanied by focal degeneration of the myocardium similar to that described for the skeletal muscle, which was affected in all but one of the embryos with myocardial changes. The affected fibers became swollen, lost their striation, and showed increased acidophilia; occasionally they stained red by the Mallory method. A few fibers showed more advanced degeneration characterized by disintegration of the sarcoplasm, nuclear pyknosis, and phagocytosis by mononuclear cells. No damage to the reticulin framework could be detected. In the youngest embryo showing myocardial changes, on the 19th day of incubation, the inflammatory cellular reaction consisted mainly of heterophilic polymorphonuclear leukocytes and myelocytes, and detectible myocardial degeneration was absent. No organisms could be found in Giemsa-stained preparations.

TITRATION OF THE VIRUS

The results of titrations of the virus are shown in Text-figure 2. The virus content of the blood and of the yolk sac reached an early peak 48 hours after inoculation of virus. Considerable, but diminishing,



Text-figure 2. Virus distribution in embryos infected with poliomyelitis virus.

amounts of virus could still be detected in the blood up to the normal time of hatching. The virus titer of the yolk sac fell more rapidly, and remained low throughout. During the first 3 days after inoculation of virus, the titer of the embryo body was considerably higher than that of the brain, and both reached a maximum on the 4th day. From then on, the titers of the body and the brain were similar, and fell slowly.

DISCUSSION

Non-Specific Lesions

None of the observed congenital abnormalities can be ascribed to the effects of virus infection. Agenesis of the eye, which was observed

1 day after inoculation of virus, could not have developed in 24 hours. Similarly, the partial dicephalus must have arisen at a very early stage of embryonic development. Hepatic necrosis similar to the lesion seen in one virus-infected embryo has been observed frequently in uninfected chick embryos.[9]

Neuronal degeneration of the tangential nucleus has not been described previously. Since no further degeneration occurred, and the number of nerve cell shadows steadily diminished during the first 5 days after the tangential nucleus could be distinguished, this is probably a normal developmental phase, similar to the cytolysis of neuroblasts in the cord which has been described by Hamburger.[14]

The ciliary, posterior root, and cranial ganglia of the 8-day chick embryo are of striking magnitude, but undergo a relative reduction in size in older embryos. This cannot be attributed to cytolysis of neurons, which is rare, but must be the result of a relatively greater rate of growth of the rest of the embryo in the later stages of development.

The pattern of Nissl substance in the ganglionic nerve cells, and to a lesser extent in the larger neurons of the central nervous system, suggests that the development of the Nissl substance in the chick embryo is not unlike Nissl regeneration in neurons recovering from poliomyelitis.[10,11] In the younger embryos only small traces of Nissl granules were present around the cell membrane; as growth proceeded, they accumulated peripherally, and in the central nervous system they finally filled the entire cytoplasm. The peripheral distribution of Nissl substance in the anterior horn cells was not observed by Sourander,[15] who used Carnoy's fluid as a fixative. Whether or not the fixative plays any part in producing this appearance, it is unlikely that the peripheral distribution of Nissl substance indicates a retrogressive process, since more advanced stages of degeneration of the affected neurons were not seen. On the other hand, the central chromatolysis and peripheral vacuolation of occasional ganglionic neurons in the later stages of development undoubtedly progressed in some instances to complete liquefaction of the cell.

Muscle lesions like those in the chick embryos have been described by Aronson and Shwartzman[16] at the site of inoculation of poliomyelitis virus into hamsters treated with cortisone. It might be argued that poliomyelitis was responsible for the slightly higher incidence of lesions in the infected embryos. Considering that the virus-infected group contained a higher proportion of older embryos, among which lesions were more numerous, it is doubtful whether the difference in incidence between the two groups is significant. Furthermore, the most severe

muscle degeneration was observed in one of the few infected embryos which showed no evidence of poliomyelitis in the nervous system. Myopathy of a similar type has been described in embryos infected with pleuropneumonia-like organisms.[17] Cover and Waller[18] have shown that spontaneous infection of chick embryos with the pleuropneumonia-like agent of chronic respiratory disease is common. No organisms could be demonstrated in the degenerating muscles in our material, and the other changes associated with this infection[17] were absent.

The occipital muscles, which were most severely affected, undergo a physiologic hypertrophy to facilitate hatching.[19,20] The predominance of the lesions in this site, especially in the older embryos, may be partially the result of the normal atrophy of the occipital muscles after hatching.

The syndrome known as "exudative diathesis" is characterized by degeneration of the skeletal muscle and myocardium of young chicks,[21] similar to that which has been described in these embryos. This disease, like myopathy of many other types in animals,[22,23] is believed to be related to vitamin E deficiency,[24] but the exact nature of the etiologic dietary factors is obscure.[21] The changes of "exudative diathesis" have been observed in other embryos and in young chicks in this laboratory, and the myopathy in the present series of embryos may well be of this nature.

Specific Lesions

Extraneural. Since myocardial degeneration is also a feature of the "exudative diathesis" of chicks,[21] the changes in the heart of the infected embryos will be considered first. Although myocarditis was not observed in the controls, it was found in only 2 of the infected embryos before the 21st day of incubation, after which time no controls were available (Table I). Degeneration of the skeletal muscle was present in all but one of the embryos with myocarditis. A detailed account of the cardiac lesions in the "exudative" syndrome is not available, but extensive myocardial degeneration has been described in "round heart disease," which is probably a variant of the same condition. In this disease, myocardial degeneration is the predominant feature and inflammation is inconspicuous or absent. The changes in the heart muscle, according to Wilson and Siller,[25] resemble the effects of avitaminosis E in calves. In contrast, the inflammatory reaction was dominant and myocardial degeneration was negligible in the embryos infected with poliomyelitis virus. The picture was essentially that of the myocarditis in human poliomyelitis.[26-28] It may be concluded tenta-

tively that the myocarditis in the embryos was not a part of the "exudative diathesis" syndrome, but was probably a direct result of infection with poliomyelitis virus.

Neural. With a few exceptions, the lesions of poliomyelitis in the chick embryo are the same as in higher animals.[10,11,29-33] Within the central nervous system, the greatest damage occurred in the brain stem and anterior horns. As in man,[11] lesions were especially severe in the motor cranial nerve nuclei, the reticular formation, vestibular nuclei, and the roof nuclei of the cerebellum (Table III), while they were virtually absent in the cortex, corpus striatum, cerebellar cortex, and sympathetic ganglia.

Lesions of the spinal sensory and the cranial nerve ganglia have been described frequently in simian[29-31,34-36] and human poliomyelitis.[36,37] Neuronal degeneration in the higher animals was never so widespread or severe as in the chick embryo, and an inflammatory cellular reaction was the predominant finding.[30,36]

The character of the lesions in the chick embryo differed in some respects from that in the higher animals. Early degeneration of neurons in primates is characterized by diffuse chromatolysis.[10] In the chick, the process was similar but the pre-existing peripheral distribution of the Nissl substance was responsible for a stage of central chromatolysis in the degenerating cells. Since peripheral chromatolysis and nuclear abnormalities are a feature of regenerating neurons,[10] it was not possible to exclude regeneration up to the 7th day after virus infection. Regenerative changes usually are not seen until much later in other species.[10] In the older embryos, all abnormal neurons exhibited the advanced nuclear degeneration and other evidence of irreversible damage,[10] so that neuronal regeneration probably did not occur.

The secondary rôle of the inflammatory reaction in poliomyelitis was demonstrated clearly by the complete absence of evidence of inflammation in the vicinity of many severely damaged neurons (Fig. 12). Anderson[38] and Dawson[39] observed a similar deficiency in relation to lesions of the central nervous system in chick embryos infected with other neurotropic viruses. The rarity of neuronophagia indicates the ability of embryonic nervous tissue to remove degenerating neurons without a cellular intermediary. Bodian[10] has suggested that the late stage of lymphocytic cuffing in poliomyelitis may be a source of antibody formation. The paucity of lymphocytic cuffing, the prolonged high levels of virus concentration (Text-fig. 2) and, possibly, the irreversibility of neuronal damage, may be correlated with the absence of

detectible circulating antibodies in the chick embryo.[40] The more pronounced fall in the virus content of the brain and body (Text-fig. 2) and the cessation of the spread of neuronal destruction after the 8th day of infection (day 15, Table III) would suggest the development of some local resistance to the progress of infection.

Since virus was inoculated into the yolk sac, the mode of infection of the embryo was comparable to the gastrointestinal route in man. The localization of lesions in the brain stem and related ganglia of the earliest embryo which showed pathologic changes suggests that entry of the virus into the nervous system may have occurred in this region, as postulated by Bodian.[41] During the acute stage of disease, when lesions were found in the ganglia, they invariably were present in the related nuclei (embryos 5 to 14, Table III). The simultaneous appearance of lesions in the nerve nuclei and ganglia provided no information as to whether centripetal or centrifugal spread of virus had occurred. However, centrifugal spread, as suggested by Bodian,[41] seems more likely, since the neurons which were nearer the affected nuclei (viz., those in the center of the ganglia) were more severely affected.

During the first 4 days after inoculation, considerable amounts of virus were demonstrated in all fractions of the embryo (Text-fig. 2), and lesions were detected in only one of 8 embryos examined. The presence, and even the multiplication of virus in a tissue need not be associated with discernible lesions. The absence of changes in the sympathetic ganglia cannot, therefore, be interpreted as evidence for or against transmission of virus by this route.

A study of the concentration of virus in the various portions of the embryo in relation to time provides some suggestive information. The virus concentration in the yolk sac and blood reached a peak 48 hours after infection (Text-fig. 2). Considering that the concentration in the brain at this time is low, it would appear that there is an extraneural phase of viral multiplication in the yolk sac or the blood. Certainly, multiplication of virus in lymphoid tissue cannot contribute, since lymphocytes do not exist at this stage. The virus concentration in the blood drops after 2 days, but rises again after the brain and embryo titers reach their maximum on the 4th to the 8th days (Text-fig. 2). There is some evidence, then, that the virus content of blood may follow passively that of the gastrointestinal tract (yolk sac), and later, that of the brain and embryo.

The close similarity between poliomyelitis in the chick embryo and in man suggests that the pathogenesis in the two species is similar, but still leaves us with the problem that "no critical evidence yet exists

which establishes the mode of spread of virus from the alimentary phase of virus multiplication to the CNS."[41]

Summary

A study is presented of the pathologic changes and the distribution of virus in the chick embryo infected by the yolk sac route with MEF1 (type 2) poliomyelitis virus.

Poliomyelitis infection in the embryo interfered with growth and sometimes was fatal.

Specific lesions were observed in the nervous system of 96 per cent of the infected embryos between the 5th and the 17th day, and in one embryo on the 3rd day, after inoculation of virus.

Neuronal degeneration was most conspicuous in the posterior root and cranial nerve ganglia, the anterior horns at all levels of the spinal cord, the formatio reticularis of the medulla and pons, the motor cranial nerve nuclei and the vestibular nucleus, the roof nuclei of the cerebellum, and the corpora quadrigemina. The gasserian ganglion was affected in all embryos showing lesions in any site.

The inflammatory response to neuronal damage was insignificant, and regeneration of nerve cells was not observed.

Myocarditis, probably caused by poliomyelitis, was present in 6 of 26 embryos showing specific lesions.

Analysis of the pathologic and virologic data suggested that the pathogenesis of poliomyelitis in the chick embryo was similar to that in man. A stage of extraneural infection during which the virus concentration reached a maximum in the yolk sac and blood was followed by a stage of neural infection characterized by high virus content and pathologic changes in the brain.

A number of non-specific lesions are described for the infected embryos, and for a series of control embryos inoculated with normal chick embryo tissue. These include congenital malformations, hepatic necrosis, neuronal degeneration, and other abnormalities in the ciliary ganglion and tangential nucleus of the vestibular nerve, and a peculiar form of myopathy in the skeletal muscle of the legs and back of the neck. The nature of these changes is discussed.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Partial dicephalus in a 12-day-old infected embryo. Of note are the two beaks and the apparent fusion of the median eyes. × 1⅓.

FIG. 2. Portion of a focus of necrosis in the liver of a 12-day-old infected embryo. Thin layer of intact cells beneath the capsule. No inflammatory reaction. Hematoxylin and eosin stain. × 200.

FIG. 3. Uninfected embryo, 11 days old. Neuronal degeneration of the bipolar neurons in tangential nucleus of the vestibular nerve. Hematoxylin and eosin stain. × 400.

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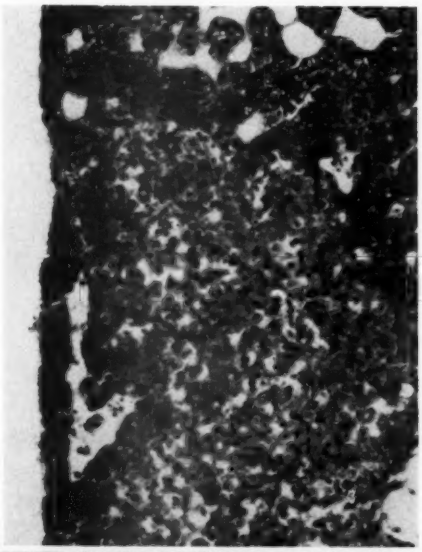

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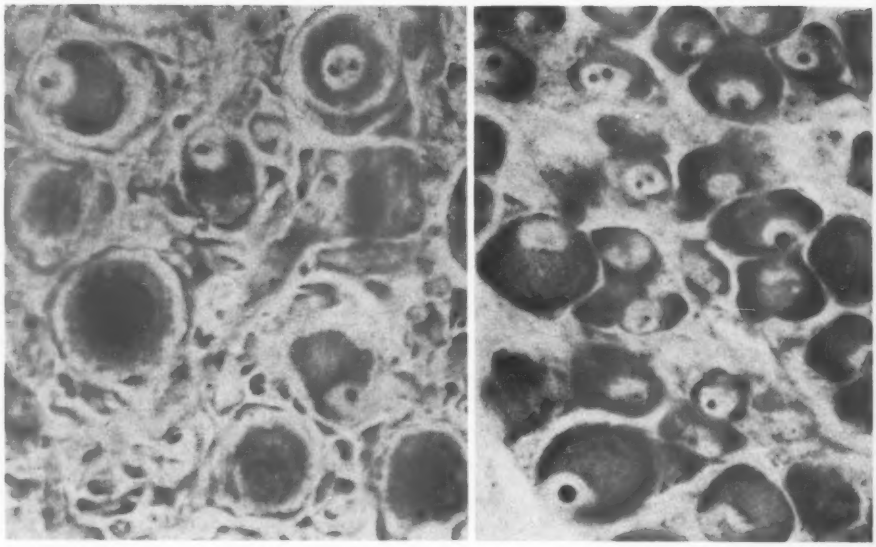


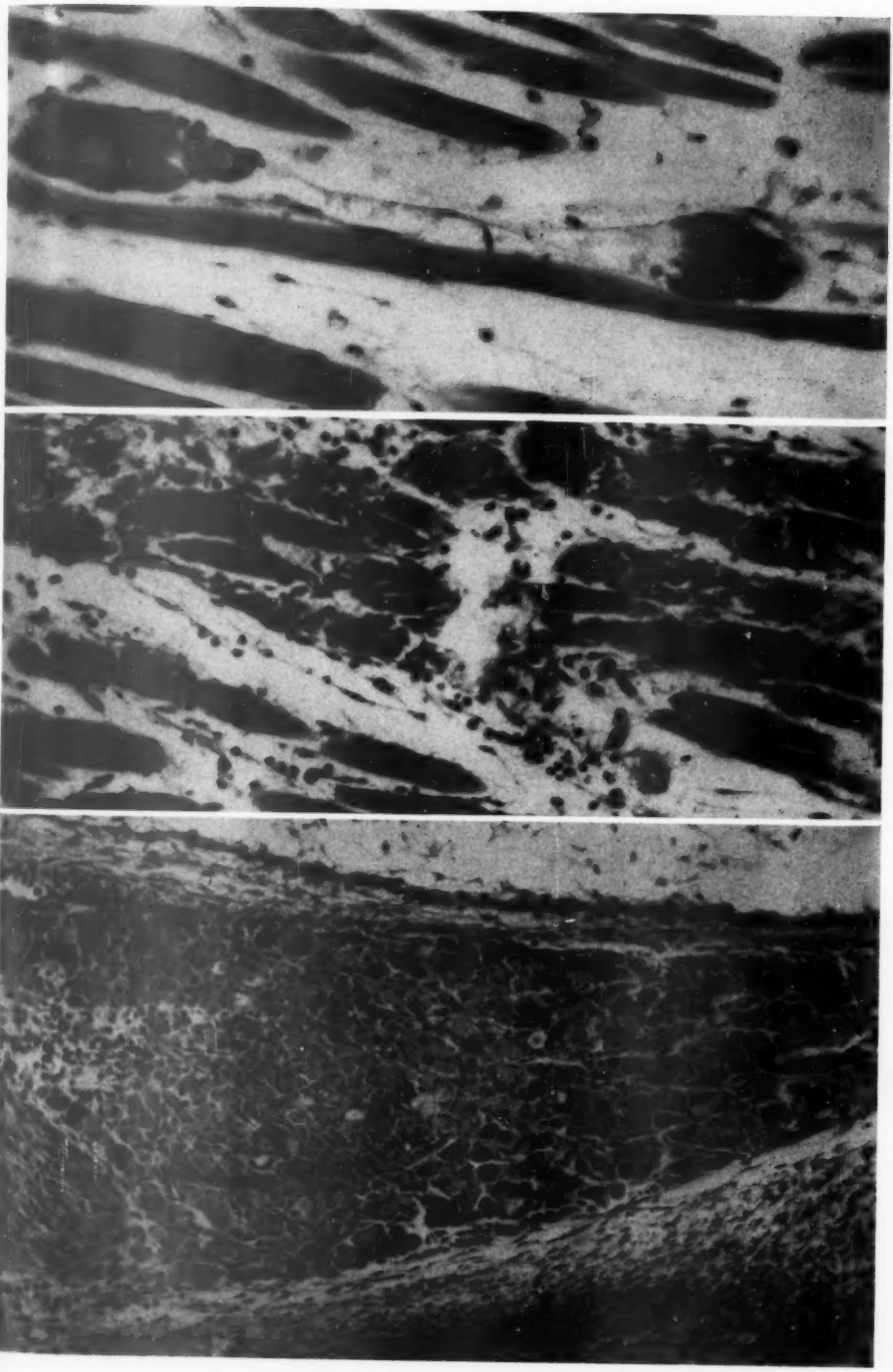
FIG. 4. Ciliary ganglion of 18-day-old uninfected embryo showing peripheral or central distribution of Nissl substance. Thionin stain. × 900.

FIG. 5. Uninfected embryo, 14 days old. Peripheral distribution of Nissl substance in neurons of posterior root ganglion. Thionin stain. × 900.

FIG. 6. Uninfected embryo, 21 days old. Swelling, increased eosinophilia, disintegration, and solution of a muscle fiber in the occipital muscle. No inflammatory reaction. Hematoxylin and eosin stain. × 800.

FIG. 7. Infected embryo, 23 days old. Inflammatory cellular infiltration of degenerating muscle fibers of the occipital muscle. Hematoxylin and eosin stain. × 200.

FIG. 8. Infected embryo, 14 days old. Severe neuronal degeneration and some perivascular inflammatory reaction in trigeminal ganglion. There is a tendency for peripheral neurons to be spared. Hematoxylin and eosin stain. × 200.



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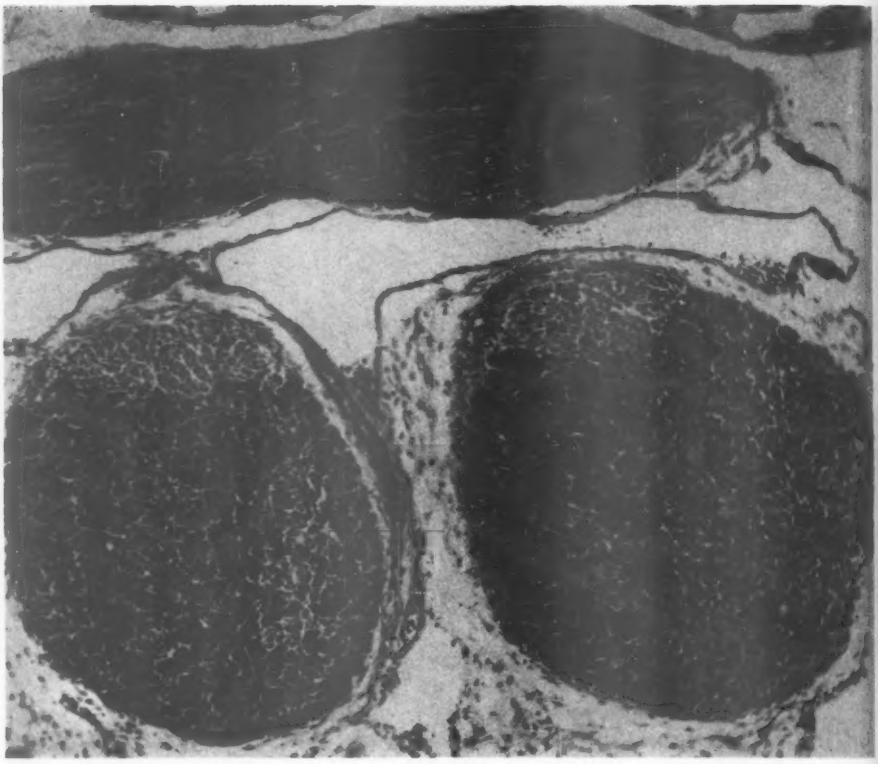
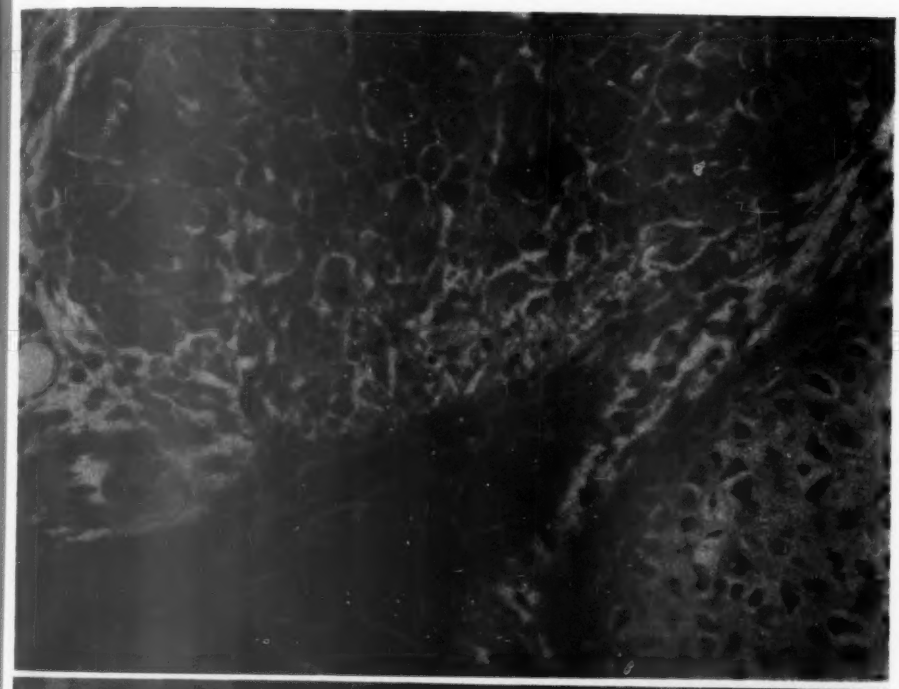


FIG. 9. Embryo, 14 days old. Neuronal degeneration in lumbar posterior root ganglia and normal abdominal sympathetic ganglion. Hematoxylin and eosin stain. × 130.

FIG. 10. Infected embryo, 12 days old. All degrees of nerve cell degeneration in brachial posterior root ganglion, with complete absence of inflammatory cellular reaction. An adjacent sympathetic ganglion appears normal. Hematoxylin and eosin stain. × 400.

FIG. 11. Infected embryo, 14 days old. Higher magnification to show neuronal damage in lumbar posterior root ganglion. Eccentric nuclei, karyolysis, and variable chromatolysis. For comparison with normal neurons in Figure 5. Thionin stain. × 900.



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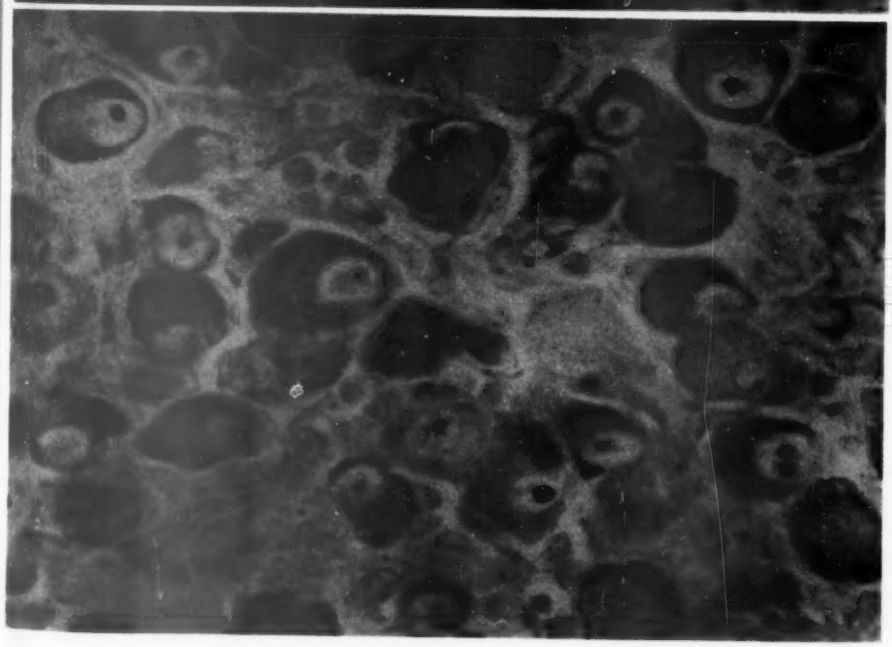

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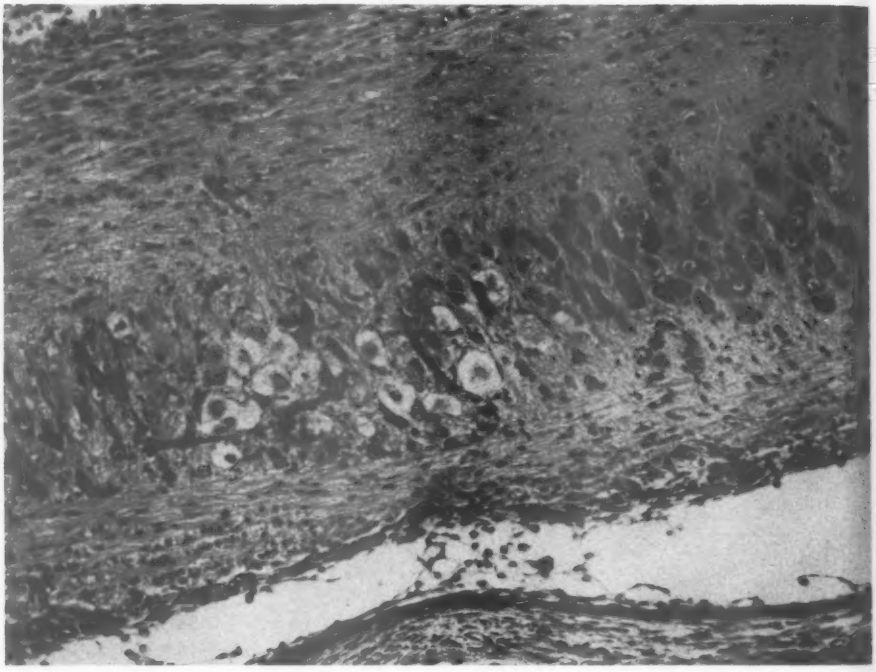
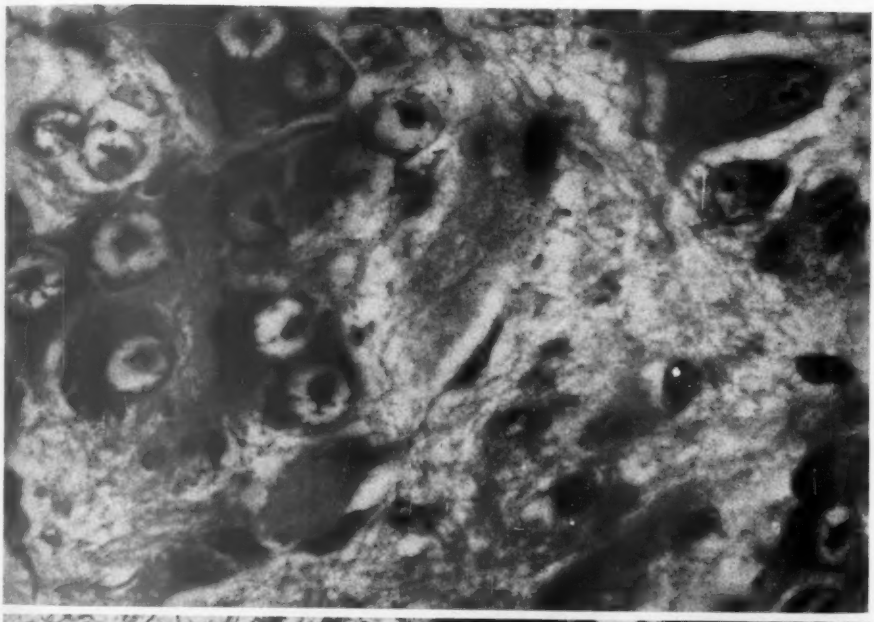


FIG. 12. Hyperacute form of neuronal degeneration of anterior horn cells in lumbosacral cord. Sagittal section of 14-day-old infected embryo. Hematoxylin and eosin stain. × 200.

FIG. 13. Infected embryo, 12 days old. Most common type of neuronal degeneration. Two intranuclear inclusions in degenerating neuron at top right. Eccentric semilunar nucleus in neuron at bottom. No inflammatory cellular reaction. Anterior horn of thoracic cord. Hematoxylin and eosin stain. × 900.

FIG. 14. Fragmentation, irregular thickening, and disappearance of neurofibrils in two anterior horn cells. Nucleus is absent in affected neurons. Lumbar cord of 15-day-old infected embryo. Holmes's stain. × 900.



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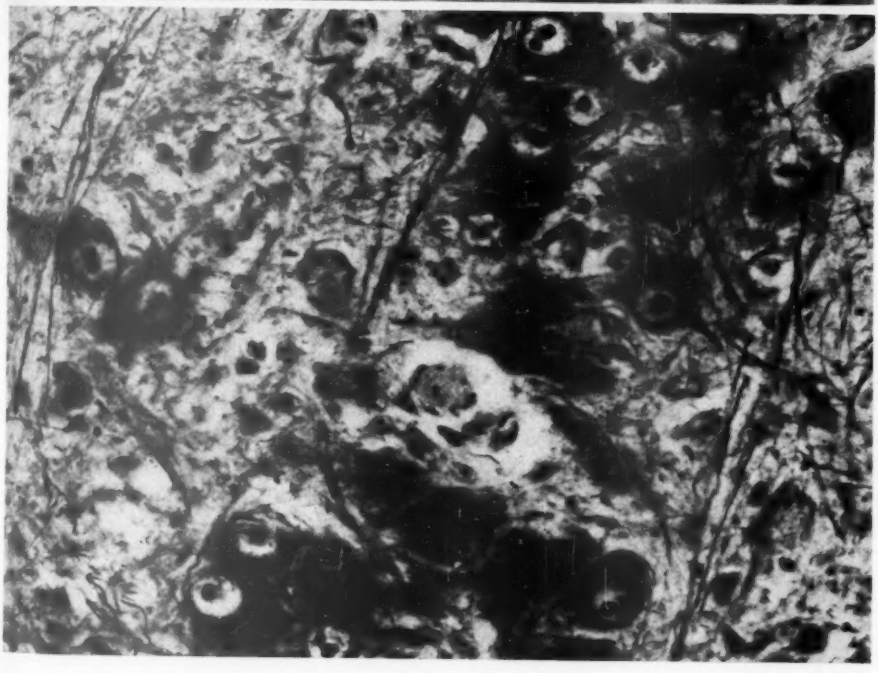

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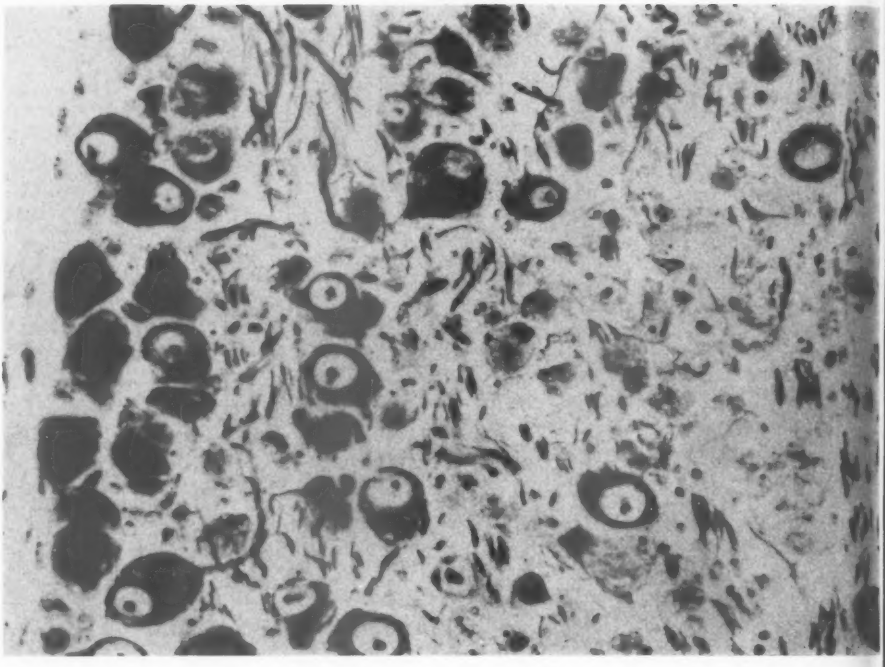
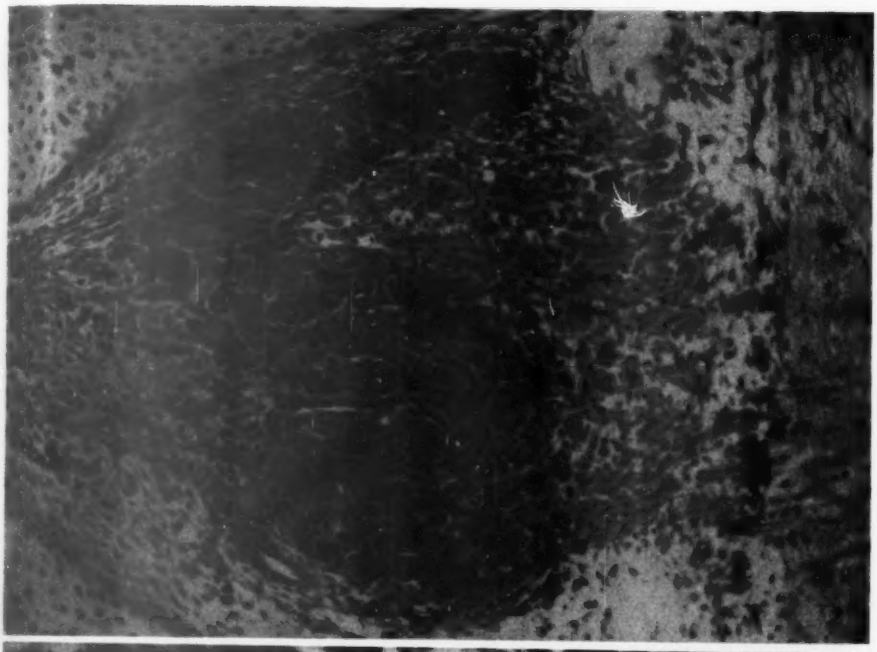


FIG. 15. Infected embryo, 14 days old. Thinning and disappearance of neurofibrils in neurons of lumbar posterior root ganglion. Neurons at periphery of ganglion (left) appear normal. Holmes's stain. × 900.

FIG. 16. Infected embryo, 12 days old. Intensely staining myeloid cells near blood vessels in and around the acoustic ganglion, which shows some neuronal degeneration. Eosin-methyl blue stain. × 200.

FIG. 17. Mononuclear inflammatory cellular reaction in myocardium of 22-day-old infected embryo. Hematoxylin and eosin stain. × 800.



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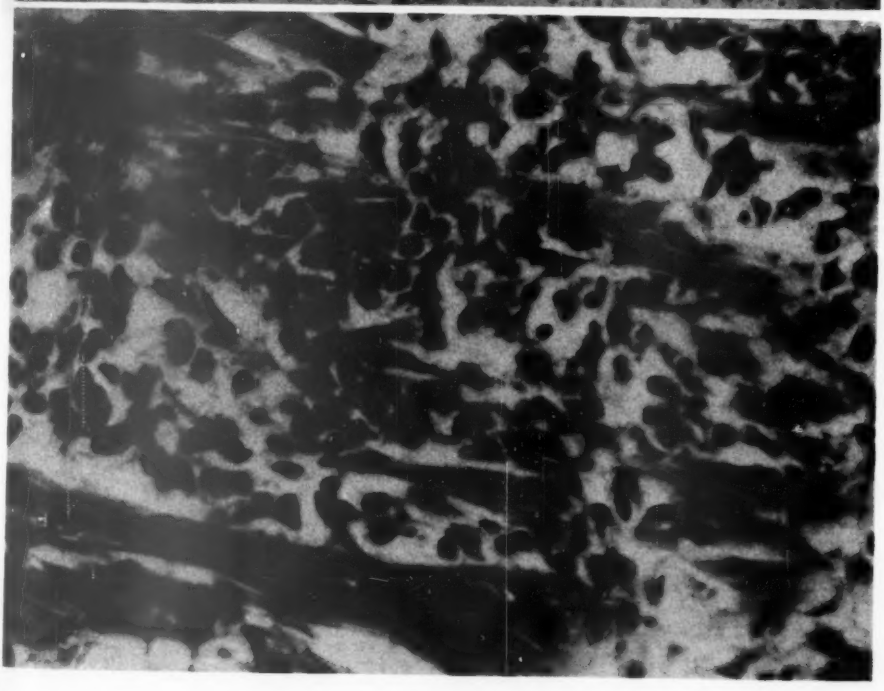

17

STUDIES ON TRICHINELLA SPIRALIS *

I. Concerning the Time and Site of Insemination of Females of Trichinella spiralis

II. Time of Initial Recovery of Larvae of Trichinella spiralis from Blood of Experimental Animals

III. Effect on the Intestinal Phase of Trichinosis of Feeding Massive Numbers of Irradiated Trichina Larvae

IV. Effect of Feeding Irradiated Trichinella Larvae on Production of Immunity to Re-Infection

V. Tests for a Strain of Trichina Larvae Resistant to Radiation

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I. CONCERNING THE TIME AND SITE OF INSEMINATION OF FEMALES OF TRICHINELLA SPIRALIS

No definite information is available as to the site, in the intestine of the host, of insemination of the adult female *Trichinella spiralis*, i.e., whether it occurs while the adult worms lie free in the intestinal lumen or while either the male or the female, or both, are partially or wholly imbedded in the mucosa. Hemmert-Halswick and Bugge[1] stated that copulation occurs deep in the mucosa of the small intestine, but they were not able to observe it. They fed trichinous meat to rats and found that at 38 hours the seminal vesicle of the male worm was filled with sperms while the vagina of the female contained none, but that at 44 hours the seminal vesicle was empty while the anterior portion of the vagina in the majority of females contained a large quantity of sperms.

* These studies were carried out (1) as part of Project No. 54 of the Michigan Memorial-Phoenix Project; and (2) under Contract No. AT(11-1)-75 for the United States Atomic Energy Commission at the University of Michigan; and (3) with the assistance of the William J. Seymour Research Fund, Wayne County General Hospital.

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They concluded, therefore, that copulation took place approximately 40 hours after feeding trichinous meat. Gursch[2] found that from 4 to 22 hours after experimental infection of rats, the trichinae were located principally in the mucosa, but that at 24 hours most of them had emerged into the lumen. He believed that this emergence might be for the purpose of copulation.

Method

In order to determine the time and site of insemination of female intestinal trichinae, white rats were tube-fed with excysted trichina larvae and then sacrificed at various intervals from 24 to 96 hours. Immediately after the animal was killed, portions of the small intestine were removed and quickly plunged into hot formalin in order to fix the worms *in situ*. Microscopic sections were made and, from selected blocks, serial sections were cut. From other portions of the intestine, adult male and female worms were removed and examined, both in the living state and after rapid fixation.

Direct observation of the adult trichinae in the small intestine of rats also was attempted between 33 and 36 hours after infection. Four animals, ranging in weight from 320 to 370 gm., were each infected with 10,000 larvae. The animals were narcotized by intramuscular injections of nembutal (0.4 ml. per 100 gm. of body weight) and an incision was made in the abdominal wall. A loop of small intestine 6 inches below the gastric pylorus was delivered, and an incision made along the antimesenteric border, bleeding being controlled by topical application of Thrombin (Parke, Davis & Co.). The animal was placed on a tray, the incised portion of the intestine resting on a piece of lucite. The exposed mucosa was examined under a dissecting microscope at a magnification of $\times$ 30.

Findings

Examination of adult trichinae removed from the intestine of infected rats revealed that insemination had taken place in a large number of female worms 33 hours after the rats were fed excysted larvae, in a few at 32 hours, and in a single worm at 30 hours. In microscopic sections of the small intestine of the infected rats, both the female and the male worms were found partially or deeply imbedded in the mucosa at 33 hours (Figs. 1 and 2). Figure 1 shows an adult male that has burrowed throughout most of its length into a villus, the posterior end (right upper portion) apparently lying outside of the villus. Figure 2 shows the anterior portion of an adult female lying

within the mucosa; the vulval opening may be seen in the left lower portion of the field. Insemination has occurred, and sperms may be seen in the forepart of the uterus. The more caudad portion of the worm is not shown in the figure; in microscopic sections of the intestine, this portion of the gravid female is often exposed in the lumen. Figure 3 shows the full length of the uterine portion of a female lying between two villi, while Figure 4 shows this portion of another inseminated female imbedded in the mucosa. Both of these sections (Figs. 3 and 4) were taken from a rat 33 hours after infection. In Figure 5 the posterior portion of a male (right) and the anterior portion of a female (left) are seen lying between two villi. The more cephalad portion of the male worm has penetrated the mucosa of a villus while the anterior portion of the female appears to be directed into the mucosa of an adjacent villus. The posterior end of the male lies in proximity to the vulval opening (not shown) of the female.

Direct observation of the mucosa of approximately 2 inches of intestine was made in each of 4 rats, for about 45 minutes, but no worms were seen in the lumen or attached to the edematous mucosa. During peristaltic movements it appeared that some fluid was expressed from the mucosa. A drop of the fluid was removed periodically from each rat by means of a capillary tube and examined microscopically. A few worms were seen in fluid from one rat infected 36 hours before. Following the direct observation of the mucosa, portions of the intestine from each of the 4 rats were placed in warm (37° C.) saline solution. When examined within 20 to 30 minutes, large numbers of extremely active worms appeared on the surface of the mucosa and were freed from the mucosa after several minutes. Microscopic examination showed that insemination had taken place in some females. Two of 10 females examined at 33 hours after infection showed a small quantity of sperm in the seminal receptacle. The seminal vesicles and vasa deferentia of 10 males examined showed large quantities of sperm. Each of 6 females examined at 36 hours had been inseminated. To date, we have not observed copulation or actual insemination in living worms or in microscopic sections.

Comment

Figures 1, 2, and 5 lend support to the belief that the male adult is imbedded throughout most of its extent in the mucosa, while a portion of the female cephalad to the vulva is also imbedded in a villus; and that the posterior end of the male and the vulval portion of the female lie between adjacent villi or in the lumen of the intestine.

Inasmuch as increasing numbers of sperms in the seminal receptacle of the females were seen at progressive intervals from 33 hours to 6 days, it seems likely that insemination occurs more than once in the same female worm. At 6 days female worms were seen with seminal receptacle distended with sperms while the forepart of the uterus contained embryos and sperms, indicating one or more inseminations; in addition, a large quantity of sperms in the vagina indicated a subsequent, very recent insemination.

Summary

White rats were fed excysted trichina larvae and were sacrificed at various intervals, from 24 to 96 hours after infection. The partially or fully developed adult forms of *T. spiralis* were removed from the small intestine and examined in the living and fixed states, and in addition microscopic sections of the small intestine of the rat were studied for evidence of the time and site of insemination. The findings indicate that insemination occurs as early as 30 hours after feeding. Our evidence suggests that during insemination a large portion of the adult male (except the posterior end) and the anterior end of the female adult are imbedded in the mucosa of villi, the cloacal portion of the male and the vulval portion of the female lying exposed between adjacent villi. Insemination of females apparently may occur more than once.

II. TIME OF INITIAL RECOVERY OF LARVAE OF TRICHINELLA SPIRALIS FROM BLOOD OF EXPERIMENTAL ANIMALS

In the symptomatology of trichinosis, it is of interest to know how early the larvae begin to invade the muscles of the host. Edney, Arbogast, and Stepp[3] (1953) stated that adult intestinal trichinae begin to produce larvae 6 to 7 days after the infection is established, i.e., after the host ingests infective larvae. However, Dunlap and Weller,[4] in 1933, found trichina embryos in focal myocardial lesions as early as 5 days after feeding and Hemmert-Halswick and Bugge[1] (1934) stated that young trichinae are born continually from the fifth day on. In all of these studies the experimental animal was the white rat. The purpose of the present study was to determine the earliest time at which young larvae of the second generation (first stage Trichinella larvae) may be recovered from the blood of experimentally infected animals of several different species. This information may be useful in indicating the approximate time when the newborn larvae begin to invade the musculature of the host.

Method

White rats, ranging in weight from 170 to 200 gm., were each fed approximately 5,000 or 6,000 excysted larvae of *T. spiralis* by stomach tube. At various periods between 96 to 120 hours thereafter, each animal was lightly anesthetized with ether. Five-tenths to 1 ml. of heparin (1:1,000 solution) was injected into the heart and a few minutes later the animal was more deeply anesthetized. After the thorax was opened, the supravalvular portion of the aorta was incised, permitting the blood to escape from the heart into the thoracic cavity. In this manner from 3 to 11 ml. of fluid blood (average, 7 ml.) was obtained from each animal. The blood was collected in test tubes and was hemolyzed by addition of four volumes of 3 per cent acetic acid. After standing for 10 minutes, the mixture was centrifuged at 2,000 r.p.m. for about 15 minutes. The supernatant was discarded and the sediment again was treated with acetic acid and centrifuged. Thin smears of the sediment were made on glass slides and allowed to dry. From 25 to 50 slides were required for examination of all of the sediment from each specimen of blood. The smears were then stained with Wright's stain and examined under a low-power lens for young larvae. This experiment was carried out by each of two observers working independently in different laboratories.

Examination also was made of blood drawn from the hearts of 5 infected rabbits. Each of 4 rabbits was fed approximately 30,000 excysted trichina larvae by stomach tube and one rabbit was fed approximately 35,000 larvae. Samples of 6 ml. of blood were withdrawn from the heart at various intervals and placed in test tubes containing dried anticoagulant (ammonium and potassium oxalate).

Four dogs (two kennel-raised beagles and two mongrels), all over 1 year of age, weighing from 22 to 27 lb. (10 to 12.3 kg.), were fed 60,000, 300,000, 150,000, and 300,000 isolated larvae, respectively, mixed with ground meat. These large doses were given because of the great resistance of dogs to trichinous infection (Matoff[5]). Five to 10 ml. specimens of venous blood were taken from each dog at each of the following intervals: 96, 110, 114, 117, 120, 144, and 168 hours. All of the dogs tolerated the infections unusually well, although 2 dogs showed transient diarrhea during the first day or two of infection.

Four monkeys (*Macacca rhesus*),* ranging in weight from 3.31 to 3.60 kg., were each fed 5,000 trichina larvae by means of a catheter passed transnasally. This dose was intermediate between a lethal dose

* These animals were made available through the courtesy of Dr. Gordon C. Brown, Associate Professor of Epidemiology, School of Public Health, University of Michigan.

(5 larvae per gm. of body weight, according to McCoy[6]) and one that produced edema, fever, and eosinophilia in monkeys (one larva per gm. of body weight, according to Welt[7]). Specimens of 5 to 10 ml. of blood were taken from the heart at the same intervals after infection, as from the dogs. One animal died on the seventh day, the other 3 were sacrificed after 11, 14, and 18 days, respectively, and the small and large bowels were examined for adult trichinae.

Altogether, approximately 3,500 smears were examined.

Results

Rats. Table I indicates that no larvae were recovered up to 110½ hours after feeding rats excysted larvae; beginning at 114 hours and

TABLE I

Number of Trichina Larvae Recovered from Blood of White Rats at Various Periods after Infection with Excysted Trichinellae

Rat no.*	Ml. of blood collected	Hours after feeding	Number of larvae recovered from blood
	Results of observer A		
1, 2	7.5, 8.5	52	0, 0
3, 4	7.5, 8	76	0, 0
5, 6	7.5, 7.5	100	0, 0
7, 8	6.5, 10	110½	0, 0
9, 10	7, 8	116	2, 18
11, 12	9.5, 11	120	14, 19
13, 14	6, 6	124	41, 66
15, 16	10, 10.5	144	150, 216
	Results of observer B		
17	5	96	0
18	5	102	0
19–22	6 (av.)	106	0
23–26	7.5 (av.)	110	0
27	4.5	114	5
28	3	116	2
29	6	118	26

* Rats 1 to 6 and 13 to 29 were each fed 5,000 excysted larvae; rats 7 to 12 were each fed 6,000 larvae.

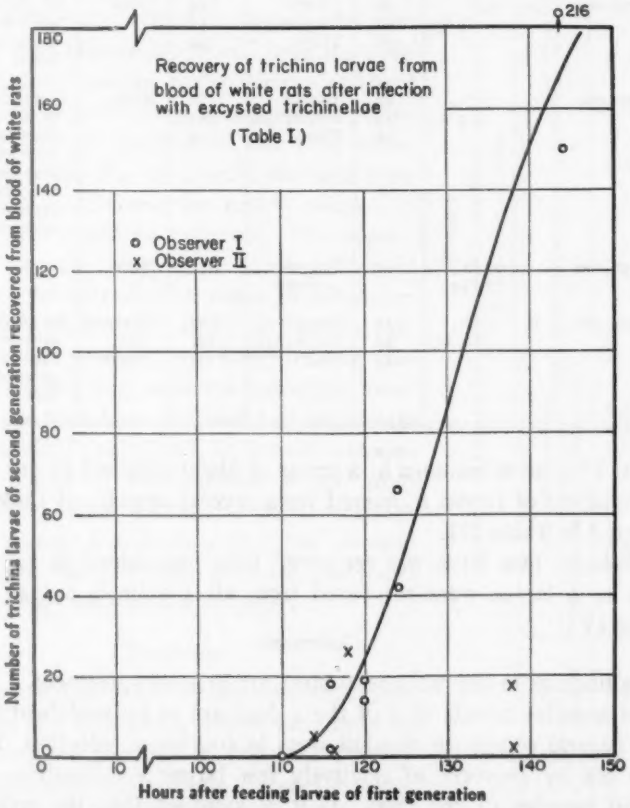
up to 144 hours, larvae were found in the blood of each of 11 rats. The average number of larvae recovered per ml. of blood at the various intervals was as follows: 114 to 116 hours, 1.2; 118 to 120 hours, 1.8; 124 hours, 9; 144 hours, 18 (Text-fig. 1).

TABLE II

Larvae Recovered from 6 ml. Blood of Rabbits after Various Intervals Following Infection

Rabbit no.*	Number of larvae recovered from blood at designated time after infection							
	96 hrs.	108 hrs.	110 hrs	114 hrs.	116 hrs.	120 hrs.	132 hrs.	144 hrs.
64	0	0				18	16	25
67	0	0				6	16	18
70	0	0				1	2	6
74	0	0				0	1	5
80			0	2	2	5		

* Rabbits 64, 67, 70, and 74 were each fed 30,000 excysted larvae; rabbit 80 was fed 35,000 larvae. Rabbit 67 (weight, 1,300 gm.) died 30 days after infection; one adult trichina was found in the intestine and 1,173,500 larvae were recovered from the muscles.



Text-figure 1. Recovery of trichina larvae from the blood of white rats after infection with excysted trichinellae (Table I).

Rabbits. Table II indicates that no larvae were recovered from the blood of 4 rabbits at 108 hours. In a fifth rabbit none were seen at 110 hours but 2 were found at 114 hours. In 6 ml. specimens of blood from these animals the average number of larvae found at 120 hours was 6; at 132 hours, 9; and at 144 hours, 13.5.

Dogs. Only one larva was found in all the smears of blood from the

TABLE III

Larvae Recovered from Muscles and from Blood of 4 Dogs Infected with Excysted Trichinellae

Dog no.	No. of larvae fed	Day of death	Weight at death	Larvae recovered from muscles			Larvae recovered from blood at 96, 110, 114, 117, 120, 144, 168 hrs.	Remarks
				Gm. of muscle	Muscle	No. of larvae		
1	60,000	30	24 lb., 10.9 kg.	38 52 68	Tongue Diaphragm Thigh	45 42 30	None	No adult trichinae in small or large bowel
2	300,000	92	27 lb., 12.3 kg.	42 46 76	Tongue Diaphragm Thigh	750 332 444	None	Biopsy (50 sections) and digestion of 2 gm. of gluteus maximus at 15 days: no evidence of old trichinosis
3	150,000		22 lb., 10 kg.		Not examined		None	
4	300,000	31	25 lb., 11.4 kg.	34 28 119	Tongue Diaphragm Thigh	624 180 1297	1 larva at 120 hours	No adult trichinae found in small or large intestine

4 dogs. This larva was seen in a smear of blood obtained at 120 hours. The numbers of larvae recovered from several muscles of these dogs are listed in Table III.

Monkeys. One larva was recovered from one animal at 120 hours and 1 to 9 larvae were recovered from all 4 animals at 144 hours (Table IV).

Comment

The findings in the rats and rabbits are in good agreement.

The negative results in 3 of the 4 dogs are to be explained by the great natural resistance of adult dogs to trichinous infection. This is borne out by recovery of relatively few larvae on digestion of the skeletal muscles of the dogs. If it is assumed that the weight of the skeletal musculature of the dog is approximately 40 per cent

of the total weight of the body, the calculated numbers of larvae in the muscles of the dogs would be: dog 1, 3,000; dog 2, 44,000; and dog 4, 52,000. In each instance, the calculated number of larvae in the muscles is considerably less than the number fed to the animal. On the other hand, in rats, guinea-pigs, and rabbits, the number of larvae recoverable from the muscles is from 100 to 1,000 times the number fed.

Because monkeys do not tolerate a heavy infection, they were given a light dose. Like the dogs, the monkeys may be regarded as having had relatively few first stage larvae circulating in the blood stream at the time the specimens of blood were obtained. Furthermore, the specimens of blood collected from the dogs and monkeys represented a small portion of the total blood volume while the specimens obtained from the rats represented the entire volume of blood that could be collected. The experiment on the dogs and monkeys would need to be extended in order to determine whether the 6-hour delay in earliest recovery of larvae (120 hours) from the blood of the dogs and the monkeys, compared to that from the blood of white rats and rabbits (114 hours), represents a slower development of reproduction in the adult female trichinae within the larger animals, or is merely a reflection of a low concentration of the first stage larvae in the blood of the dogs and monkeys tested.

TABLE IV

Number of Trichina Larvae Recovered from Blood of Rhesus Monkeys at Various Periods after Infection

Monkey no	Day killed or died (d)	Adult trichinae recovered		Larvae recovered from blood after designated time						
		Small intestine	Large intestine	96 hrs.	110 hrs.	114 hrs.	117 hrs.	120 hrs.	144 hrs.	168 hrs.
1	d 7	119	0	0	0	0	0	0	2	1
2	11	467	9 females	0	0	0	0	0	9	6
3	14	1348	1 male, 5 females	0	0	0	0	1	2	2
4	19	341	2 females	0	0	0	0	0	1	5

Summary

Twenty-nine adult white rats, 5 rabbits, 4 dogs, and 4 rhesus monkeys were infected with counted numbers of excysted Trichinella larvae. At various intervals ranging from 96 to 168 hours after infection, specimens of venous or cardiac blood were

examined for first stage larvae. In the rat and rabbit, first stage Trichinella larvae were recovered earliest at 114 hours after negative results at 110 hours; in the dog and monkey, larvae were recovered earliest at 120 hours after negative results at 117 hours.

Conclusion

First stage larvae of *T. spiralis* are present in the blood of white rats, rabbits, dogs, and rhesus monkeys in the latter part of the fifth day after infection of these animals with excysted larvae.

III. EFFECT ON THE INTESTINAL PHASE OF TRICHINOSIS OF FEEDING MASSIVE NUMBERS OF IRRADIATED TRICHINA LARVAE

In previous experiments[8] it was shown that a dose of 18,000 r. of cobalt-60 applied to trichinous muscle prevented over 99 per cent of larvae of *T. spiralis* from developing to adult worms, when the irradiated larvae were fed to white rats. This dose, however, does not kill the larvae and one may ask if a sufficiently large number of muscle larvae present in pork exposed to this dose of cobalt-60, upon being ingested with the meat, would produce irritation of the intestinal tract (enteritis) and ill effects in the host; or, specifically, if a person were to eat raw or undercooked trichinous pork that had been subjected in all parts to a dose of at least 18,000 r. cobalt-60, would it be likely that he would suffer ill or serious effects as a result of intestinal irritation by the ingested larvae? An indirect answer to this question was sought in the present experiments on the effects of feeding rats irradiated larvae in massive numbers equal to or greater than the number of non-irradiated larvae that cause fatal infection.

This investigation is in line with a recommendation included in the report of the Second National Conference on Trichinosis[9] that a study be conducted on the effect of gamma irradiation of trichinous meat on the enteric phase of trichinosis. The results of such a study should be of interest in connection with the proposal[10] that all raw pork be exposed to gamma radiation as a practical means of controlling trichinosis in man and pig.

Experiment 1. Effect of Feeding 10,000 Non-irradiated Larvae Upon Survival of Rats

McCoy[11,12] found that the minimal lethal dose of trichina larvae that usually kills white rats is about 40 per gm. of body weight. A preliminary experiment was carried out to test the effect of feeding

10,000 non-irradiated larvae upon survival of 6 white rats weighing 125 to 150 gm. (67 to 80 larvae per gm. of body weight) for 30 days.

Results. Three of the animals died within 13 days and all showed evidence of having had diarrhea. Three survived the test period of 30 days, and 2 of these showed evidence of diarrhea. In one animal that died after 4 days, 10,000 adult worms were recovered from the small bowel; in one that died after 6 days, 6,400 adult worms were found in the small intestine, and in one rat that died after 13 days, 5,000 adult worms were recovered. The remaining 3 rats were sacrificed on the 30th day. On examination of the contents of the small intestines, 0, 1,800, and 3 adult trichinae, respectively, were found; and on digestion of eviscerated, skinned carcasses, 73,920, 128,520, and 75,600 muscle larvae, respectively, were recovered.

Summary. Feeding of 10,000 non-irradiated trichina larvae to 6 rats (weight, 125 to 150 gm.) resulted in diarrhea in 5 rats and in death of 3 of the 6 rats during the intestinal stage.

Experiment 2. Effect of Feeding Rats with 12,000 Larvae Exposed to 10,000 r. Cobalt-60

In a second experiment, trichina larvae were obtained by digestion of the ground muscles of a white rat that had been infected 3 months previously. A portion of the yield of larvae was exposed ***in vitro*** in tap water in a lusteroid tube to 10,000 r. cobalt-60 from a kilocurie source (radiation rate, 1,333 r. per minute). The remaining larvae were not irradiated. This dose of cobalt-60 (gamma rays) applied to trichina larvae ***in vitro*** produces sexual sterilization of most of the developing adult worms.[8] Inasmuch as the rats available for this experiment weighed more than those in experiment 1, it was decided to increase the number of larvae to be fed to each rat to 12,000. In one group of 12 rats (weight, 200 to 250 gm.), each was fed 12,000 non-irradiated larvae (48 to 60 larvae per gm. of body weight), presumably a lethal infective dose; and in another group of 12 rats of the same weight, each was fed 12,000 larvae that had been irradiated with 10,000 r. cobalt-60.

Results. All 12 rats fed non-irradiated larvae (Table V) died within 24 days (4 at 2 days, 1 at 12 days, 2 at 14 days, 2 at 15 days, 1 at 16 days, 1 at 18 days, and 1 at 24 days; average length of life, 11 days). The average weight of these rats at the time of feeding was 238 gm. and at the time of death, 140 gm. The average loss of weight, therefore, was 98 gm., representing 41 per cent of the average original weight. In all rats the loss of weight was greatest during the first week

TABLE V

Effect on Intestinal Phase of Trichinosis of Feeding Rats Massive Numbers (12,000 or 24,000) of Trichina Larvae Exposed to 10,000 r. or 18,000 r. Cobalt-60

Exp. no.	No. of larvae fed	Dose Co^{60} to larvae	No. of rats	Day rats died (d) or were killed (k)	No. of rats with diarrhea	Av. % loss (−) or gain (+) in weight after 1 wk.	No. of adult trichinae recovered from intestine of rats	No. of larvae recovered on digestion of muscles (after 17th day)
2	12,000	0	12 control (4)	d 2	10		4,000–7,020, av. 5,246	—
			control (7)	d 12–18			4,136–10,000, av. 6,925	4,600 (18 da.)
			control (1)	d 24			3,250	197,000
	12,000	10,000 r.	12 test	k 31	6		11 rats, 0; 1 rat, 2	0–620, av. 226
3	12,000	0	3 control	d 11, 12, 21	3	−27	170, 7,360, 998, av. 2,843	—, —, 187,000
	12,000	18,000 r.	10 test (2)	d 7, 7	0	+ 9	0	—, —
			test (8)	k 30		+ 6	0	6 rats, 0; 1 rat, 12; 1 rat, 45
4	24,000	0	3 control	d 5, 6, 13	3	−21	7,000, 7,650, 3,400, av. 6,017	—, —, —
	24,000	18,000 r.	10 test (1)	d 4	1	−16 (4 da.)	0	—
			test (1)	k 8	0	− 3	3	—
			test (8)	k 43	0	+14	0	8 rats, 0
5	12,000	0	15 control (12)	d 2–6	9		1,470–12,000, av. 7,255	—
			control (1)	k 12			5,160	—
			control (1)	d 15			6,176	—
			control (1)	d 25			350	54,000
	12,000	10,000 r.	15 test (3)	k 6	4		700–3,129, av. 2,273	—
			test (1)	d 12			1,080	—
			test (2)	k 12			1,050, 2,600 av. 860	—
			test (3)	k 18			1, 44, 382	0, 0, 0
			test (1)	d 19			594	6
			test (3)	k 24			0, 0, 5	136, 506, 980 av.
			test (2)	k 33			0, 0	588, 1,259 694

(average loss, 26 per cent) but continued in successive weeks until death. Necropsy revealed adult worms in the small intestine of all rats, the number of adult trichinae recovered ranging from 3,250 to 10,000 (average number recovered, 6,060). No muscle larvae were recovered in any of the rats dying on the 16th day or earlier, inasmuch as muscle larvae do not resist digestion until the 18th day or later. In the rat that died on the 18th day, 4,600 muscle larvae were recovered and in the animal that died on the 24th day, approximately 197,000 larvae were recovered from the muscles. All but 3 rats showed evidence of diarrhea within 48 hours; one of these 3 had diarrhea at 6 days, and in 2 no indication of diarrhea was observed.

All of the 12 rats that were fed irradiated larvae survived the test period of 31 days. Evidence of diarrhea, however, was noted in 6 rats (4 at 3 days, 1 at 5 days, and 1 at 7 days), but all of these soon recovered and by the eighth day appeared well. The average weight of the rats was 223 gm. at the time of feeding, 171 gm. 1 week later, 178 gm. 2 weeks later, and 230 gm. at the time of sacrifice 31 days after feeding. All 12 rats lost weight during the first week, the average loss being 52 gm. (23 per cent). The animals began to regain weight during the second week, and at 31 days had recovered their original weight and appeared active and healthy. No gross pathologic changes were noted at necropsy. Adult trichinae were recovered from the small intestine of only one of the 12 rats and in this rat only 2 adult worms were found (Table V). Trichina larvae of the second generation were recovered from the skeletal muscles of 10 of the 12 rats, the number of muscle larvae ranging from 0 to 620. The average number of the larvae recovered from the muscles in the 12 rats was 226.

Summary. Twelve control rats all died following an infective dose of 12,000 non-irradiated larvae (48 to 60 larvae per gm. of body weight). All 12 animals developed severe dehydration and marked loss of weight, and in 10 evidence of diarrhea was observed.

Twelve test rats, fed the same number of larvae that had been irradiated with 10,000 r. cobalt-60, survived a 31-day test period. These rats developed, at most, only mild irritation of the intestinal tract (transient diarrhea in 6 of 12 rats) and only slight loss of weight during the first week of infection.

Exposure of trichina larvae *in vitro* to 10,000 r. cobalt-60 prevented reproduction in most of the developing adult trichinae. In 12 rats, on the average, only 226 muscle larvae of the second generation were recovered, compared to an expected recovery ranging from 100,000 to 500,000 in rats fed the same number of non-irradiated larvae.

Experiment 3. Effect of Feeding Rats 12,000 Larvae Exposed to 18,000 r. Cobalt-60

Experiment 2 was repeated but the amount of irradiation applied to the larvae was increased to 18,000 r. Ten test rats (average weight, 171 gm.) were each fed 12,000 larvae (average, 70 larvae per gm.) that had been exposed to 18,000 r. cobalt-60, and 3 control rats (weighing 173, 172, and 182 gm.; average, 176 gm.) were fed the same number of non-irradiated larvae. During the first week the control rats showed evidence of diarrhea, appeared sluggish, did not respond readily to mechanical stimulation, and lost weight to 110, 102, and 172 gm., respectively (average, 128 gm.; average loss of weight, 27 per cent). One of the 3 control rats died on the 11th day, one on the 12th day, and the third on the 21st day; their intestinal contents had 170, 7,360, and 998 adult worms, respectively.

None of the 10 test animals showed any evidence of diarrhea, sluggishness, or lack of reactivity to mechanical stimuli, and none showed any loss of weight. Actually, after 1 week, the animals had an average gain in weight of 7 per cent. Two of the animals died after 7 days from causes apparently unrelated to trichinous infection. At the time of death, neither of the 2 that died at 7 days and none of the 8 that were sacrificed at 30 days revealed any adult worms in the intestinal tract. In 2 of the 8 that survived for 30 days, a few (12 and 45) larvae were recovered on digestion of their muscles. On the other hand, the control rat that died 21 days after being fed non-irradiated larvae yielded 187,000 larvae on digestion of its muscles.

Summary. No evidence of diarrhea was observed in any of 10 rats fed 12,000 trichina larvae (average, 68 larvae per gm. of body weight) exposed to 18,000 r. cobalt-60, a dose of irradiation that prevents the vast majority of larvae from maturing to adult forms. Despite the large number of larvae fed, the irradiated larvae and the adult forms that developed from them did not induce diarrhea and were quickly lost from the intestinal tract of the host.

Experiment 4. Effect of Feeding Rats 24,000 Larvae Exposed to 18,000 r. Cobalt-60

Experiment 3 was then repeated, feeding 24,000 instead of 12,000 larvae. Three control rats were fed non-irradiated larvae and 10 test rats were fed larvae exposed to 18,000 r. cobalt-60. All 3 control rats (average weight, 151 gm.; average number of larvae fed, 159 per gm.) developed severe diarrhea and died from intestinal effects of the infection. One died on the 5th day, one on the 6th day, and one on the

13th day. The average weight at death was 109 gm. The number of adult trichinae recovered from the small intestines of these rats was 7,000, 7,650, and 3,400, respectively.

One of the test rats, apparently in good condition, was sacrificed on the eighth day in order to examine the small intestine for adult trichinae. Three adult worms were recovered, 2 of which were females and one male; one of the females contained embryos and one showed no sperms in the receptaculum seminis.

Of the remaining 9 test rats (average weight, 147 gm.; average number of larvae fed, 163 per gm.), only one developed diarrhea and this animal died on the fourth day. No adult worms, however, were found in the intestinal tract of this rat. The other 8 test rats gained steadily in weight, their average initial weight being 144 gm. before infection, 164 gm. 1 week after infection, and 251 gm. 43 days after infection. No adult trichinae were recovered from the small intestine of any of these 8 rats.

Comments. As would be expected from the previous findings in experiment 3 in which the 3 control rats died (11 days, 12 days, and 21 days, respectively; average, 15 days) after being fed 12,000 non-irradiated larvae, in the present experiment the 3 control rats, fed 24,000 non-irradiated larvae, died sooner (5 days, 6 days, and 13 days, respectively; average, 8 days).

The cause of death in one test rat that died on the fourth day after being fed non-irradiated larvae is not clear; this rat developed diarrhea and lost 27 gm. in weight from the time of infection until death, but a thorough examination of the intestinal contents at the time of death, 4 days after infection, did not reveal the presence of any adult trichinae. In the test rat sacrificed at 8 days only 3 adult worms were recovered, compared to the average of 6,016 adults recovered from the 3 control rats that died on the 5th, 6th, and 13th days.

Summary. Nine of 10 rats, each fed 24,000 larvae exposed to 18,000 r. cobalt-60, showed no evidence of diarrhea and their weight increased steadily. In all 10 test rats the trichinae disappeared rapidly from the small intestine.

Experiment 5. Do Irradiated Larvae Disappear from the Intestine of Rats More Rapidly than Non-irradiated Larvae?

To answer this question, experiment 2 was repeated employing 30 rats, each of 15 test animals being fed 12,000 larvae irradiated with 10,000 r. cobalt-60 and each of 15 control animals being fed 12,000 non-irradiated larvae. The rats ranged in weight from 200 to 250 gm.;

the number of larvae fed was 48 to 60 per gm. of body weight. It was planned to sacrifice 3 rats of each group at intervals of 6 days. At death the intestinal contents of the rats were examined for recovery of adult worms. All animals were weighed and their appearance noted at the beginning of the experiment and at the time of death. At necropsy, tissue was taken from the tongue, heart, lungs, diaphragm, liver, spleen, small intestine, and skeletal muscles for microscopic section for evidence of trichinous infection or other disease. From rats surviving for 18 days or more, the muscles were macerated and digested for the presence and number of muscle larvae.

Results. Of the 15 rats fed non-irradiated larvae, all but one died within 25 days (3 at 2 days, 6 at 3 days, 1 at 5 days, 2 at 6 days, 1 at 15 days, and 1 at 25 days; average number of days of survival, 6); the remaining animal was sacrificed at 12 days. Among 12 rats that died within 12 days, the average number of adult worms recovered was 7,255 (Table V). In the rat that survived to the 25th day, only 350 adult worms were recovered from the intestine and 54,000 larvae were recovered on digestion of the muscles.

Of the 15 rats that were fed irradiated larvae, 2 died (1 at 12 days and 1 at 19 days). The others were sacrificed at various intervals (3 at 6 days, 2 at 12 days, 3 at 18 days, 3 at 24 days, and 2 at 33 days). From the 6 rats that were examined at 6 or 12 days, 1,925 adult worms, on the average, were recovered from the small intestine; of 4 rats examined at 18 or 19 days, the average of the numbers of adult worms found was 155; of the 5 rats examined at 24 or 33 days, 4 had no adult worms and 1 had 5 adults. The average number of trichina larvae recovered on digestion of the muscles of these 5 rats was 694.

After white rats were fed 12,000 trichina larvae, the average number of adult worms recovered from the small intestine 12 to 18 days after feeding was 6,925 in animals fed non-irradiated larvae (9 control rats in experiments 1 and 2), and 860 in those fed larvae that had been irradiated with 10,000 r. cobalt-60 (6 test rats in experiment 2).

Summary. When trichina larvae irradiated with 10,000 r. cobalt-60 were fed to 15 white rats, the developing intestinal forms disappeared more rapidly from the small bowel at all intervals examined (from 6 to 33 days) than adult worms that developed from non-irradiated larvae fed to 15 control rats. A few irradiated larvae were able to develop sexually and reproduce, since small numbers of larvae (average, 694 larvae) of the second generation were recovered on digestion of the muscles of 5 experimental animals 24 to 33 days after infection.

General Summary

White rats fed 12,000 non-irradiated excysted larvae of *T. spiralis* (48 to 60 larvae per gm. of body weight) died, apparently mainly as a result of the intestinal phase of the infection. The feeding of the same number of larvae that had been exposed to 10,000 r. cobalt-60 merely caused transient diarrhea in some animals. The feeding of 12,000 larvae or of 24,000 larvae that had been exposed to 18,000 r. cobalt-60 generally (in 19 of 20 rats) caused no diarrhea and permitted the animals to gain steadily in weight.

In all rats fed irradiated larvae (10,000 r. or 18,000 r.), the parasites were lost rapidly from the intestinal tract.

It is inferred, therefore, that if a person were to eat raw or undercooked, heavily trichinosed pork that was irradiated in all parts with at least 18,000 r. cobalt-60, he would suffer little or no ill effect from irritation of the intestine by the larvae, and that the parasites would disappear rapidly from the intestinal tract.

IV. EFFECT OF FEEDING IRRADIATED TRICHINELLA LARVAE ON PRODUCTION OF IMMUNITY TO RE-INFECTION

In 1942, Levin and Evans[13] induced immunity in rats to re-infection by *T. spiralis* by feeding the rats larvae that had been irradiated with 3,250 to 3,750 r. of x-ray. This amount of x-radiation allowed the larvae to grow to maturity but most of the adult worms were sterile, so that very few larvae of the second generation were recoverable from the muscles of the host. These observers concluded that the origin of the mechanism of immunity against re-infection is located in the intestine.

The purpose of the present experiment was to determine if rats initially infected with larvae that had been irradiated with cobalt-60 would develop immunity to re-infection with non-irradiated larvae. The following doses of cobalt-60 were applied to the larvae: (1) 10,000 r., which produces sexual sterilization of most of the adult worms, and (2) 18,000 r., which prevents most of the larvae from maturing to adult forms. The findings of this experiment should be of interest in connection with the possible development of immunity to trichinosis in man as a result of eating trichinous pork irradiated with cobalt-60 (Gould, Gomberg, and Bethell[10]).

Method

Eight groups of white rats were employed ranging in weight from 150 to 175 gm. Each rat was fed 3,700 trichinae (average, 20 to 22 larvae per gm. of body weight). At the time of death of the animals, the number of intestinal trichinae was determined; in rats that lived more than 17 days after a primary infection, the number of larvae in the skeletal muscles also was determined. Each group of rats was divided into two lots and subsequent observation and examinations on each lot were carried out independently by workers in each of two different laboratories.

Group I (control) consisted of 15 rats each given a single infection with 3,700 non-irradiated larvae.

Groups II (12 rats), III (7 rats), and IV (12 rats) were re-infected with non-irradiated larvae 40 days after receiving an initial infection with (a) non-irradiated larvae, (b) larvae exposed to 10,000 r. cobalt-60, and (c) larvae exposed to 18,000 r. cobalt-60, respectively. Group II served as a control on immunity developed through an ordinary infection.

Group V (control) consisted of 12 rats each fed 3,700 non-irradiated larvae from the batch used to re-infect rats of Groups II, III, and IV.

In groups VI (17 rats), VII (15 rats), and VIII (17 rats) the same scheme was followed as in groups II, III, and IV except that rats of group VII initially received larvae exposed to 6,000 r. and those of group VIII initially received larvae exposed to 12,000 r. cobalt-60. Group VI was a control like group II, in which the animals were infected initially with non-irradiated larvae. As before, 40 days after the initial infection each rat in all three groups was re-infected with 3,700 non-irradiated larvae.

Findings and Comments

Table VI indicates the results. (Also see Text-fig. 2.)

Two rats of group I (controls with single infection) had some adults in the intestine at 15 days and one of 2 rats had a few trichinae at 20 days. These findings are in agreement with those expected, inasmuch as adult worms generally disappear by the 16th day from the small intestine of rats fed a moderate number of larvae (up to 20 larvae per gm.[14]).

Rats of group II (re-infected controls) showed relatively few or no trichinae at the various intervals examined. Thus, of 7 rats sacrificed between 4 and 10 days after infection, only 3 had any larvae (8, 4, and 52). The average number in these 7 rats was 9 compared to an average

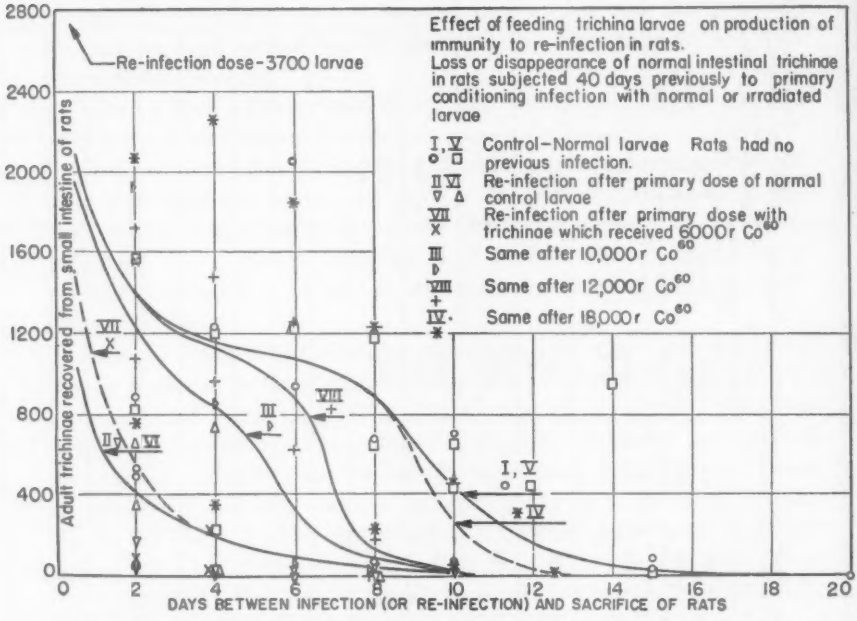
TABLE VI

Experiment 2. Effect of Feeding Rats Irradiated Trichina Larvae on Production of Immunity to Re-infection (Infective Dose 3,700 Larvae).
(1) Rate of Disappearance of Intestinal Trichinae, (2) Number of Muscle Larvae Recovered from Rats, after Primary Infection and after Re-infection

Days after infection or re-infection that rat was sacrificed*	Group I Control rats fed non-irradiated larvae		Test rats initially fed larvae exposed to designated amount of Co^{60}; re-infected after 40 days with non-irradiated larvae						Group V — Control rats infected with same lot of larvae used to re-infect rats of groups II, III, IV		Rats initially fed larvae exposed to designated amount of Co^{60}; re-infected after 40 days with non-irradiated larvae		
			Group II — No Co^{60}		Group III — 10,000 r. Co^{60}		Group IV — 18,000 r. Co^{60}				Group VI No Co^{60}	Group VII 6,000 r. Co^{60}	Group VIII 12,000 r. Co^{60}
	Adult trichinae in intestine	Larvae recovered from muscles	Adult trichinae in intestine	Larvae recovered from muscles	Adult trichinae in intestine	Larvae recovered from muscles	Adult trichinae in intestine	Larvae recovered from muscles	Adult trichinae in intestine	Larvae recovered from muscles	Number of adult trichinae recovered from the small intestine		
2	883 525 495		17 165	181,000 32,300	1,912	15	2,060 756	– 0	1,576 816		63 357	75 1,567	1,066 1,705
4	1,226 1,177		0 8	166,000 25,800	848	11	357 2,248	18 2	208 1,187		1 733	21 230	951 1,479
6	2,100 871		0 4	164,000 47,240	42	0	939 1,844	0 0	1,211		2 1	0 0	623 1,248
8	667		52 0	101,000 640,000	9	0	220 1,240	27 3	1,185 622		100 0	0 0	17 760
10	700 72		0	156,000	0	22	545 14	0 –	649 436		0 0	0 0	186 0
12												0	
13							2	–					
14									952				
15	94 40		0 0	190,000	0	0			11		5 0	0 0	12 0
18												0	
20	0 6	18,000 23,400	0	96,000	0	496	0	28,000	1	3,000 53,000	0,0 0,0	0	0 0
25											0		0,0,17
34	0	158,000											

*** Rats of groups I and V received only one infection and were sacrificed on the indicated day after infection; all other rats were infected twice and were sacrificed on the indicated day after re-infection.**

of 973 in 7 rats of group I sacrificed during the same period. This finding indicates that the primary infection with non-irradiated larvae in rats of group II was effective in establishing a high degree of immunity to re-infection (see McCoy[11]). McCoy[14] found that in rats rendered immune to trichinosis, subsequent feeding of larvae resulted in rapid loss of the trichinae from the intestines 8 to 18 hours after feeding.



Text-figure 2. Effect of feeding trichina larvae on production of immunity to re-infection in rats.

Rats of group III similarly showed that some evidence of immunity was established by feeding larvae exposed to 10,000 r. cobalt-60. The number of adult worms recovered from the intestine appeared to be diminished in the rats sacrificed 6, 8, and 10 days after infection, as compared to rats of group I. It is recognized, of course, that the number of test animals is too small to permit definite conclusions to be drawn.

On the other hand, no reduction was noted in the number of intestinal trichinae recovered from rats of group IV, fed with larvae irradiated with 18,000 r. cobalt-60.

The findings in group V (controls with single infection) are in essential agreement with those in group I.

The findings in group VI (re-infected controls) generally are in agreement with those of group II, a similar control group. No adult worms were found in the intestine of any of 11 rats of group VII 6 days after re-infection, as a result of feeding them initially with larvae exposed to 6,000 r. cobalt-60. On the other hand, rats of group VIII, initially fed larvae exposed to 12,000 r. cobalt-60, showed relatively little reduction in number of adult worms 6 to 10 days after re-infection, compared to rats of group I.

Conclusion

Rats fed larvae of *T. spiralis* that have been exposed to 10,000 r. cobalt-60 (a dose of radiation that does not prevent the larvae from developing into adult forms but does result in partial or complete sexual sterilization of the adult worms), develop a definite degree of immunity to re-infection with non-irradiated larvae. If the dose of irradiation with cobalt-60 is increased to 18,000 r. (a dose that prevents most larvae from maturing to adult forms), little or no immunity results.

V. TESTS FOR A STRAIN OF TRICHINA LARVAE RESISTANT TO RADIATION

Variation in susceptibility of different trichina larvae to the effects of x-radiation was first noted by Schwartz.[15] In a previous work[16] we found that small percentages of larvae withstood the respective dose of x-ray that generally produced (1) loss of motility of larvae, (2) inhibition of maturation of larvae to adult forms, or (3) sexual sterilization of the adult worms developing from the irradiated larvae. Similar observations[8] were made by us on larvae exposed to cobalt-60. A possible explanation for the ability of a small percentage of larvae to withstand a dose of x-ray or of cobalt-60 that produces sexual sterilization in most of the developing adult trichinae is that the radiation does not hit or damage the sensitive sites in the gonadal cells of the unaffected larvae. The possibility that exposure of trichina larvae to gamma radiation might result in selective survival of those which were relatively radioresistant and that this might conceivably lead to a race of radioresistant larvae, was suggested to us by Dr. Carl V. Weller. To check this possibility, successive generations of larvae were irradiated with the same dose of cobalt-60 and then tested for their productivity.

Method

Initially, 5 groups of rats were employed, each rat being tube-fed 5,000 larvae. Group I (control) consisted of 5 rats fed non-irradiated

larvae; group II, 12 rats fed larvae irradiated with 50,000 r. cobalt-60; group III, 12 rats fed larvae irradiated with 30,000 r.; group IV,A, 12 rats fed larvae irradiated with 18,000 r.; and group V,A, 10 rats fed larvae irradiated with 10,000 r. cobalt-60.

Approximately 2 months after infection, the rats were sacrificed

TABLE VII

Tests for Strain of Trichina Larvae Resistant to Cobalt-60. Effect of Cobalt-60 Applied to Larvae in Vitro *on Reproduction in Succeeding Generations of Trichina*

Group no.	No. of rats*	Dose of Co^{60}, r.	No. of larvae recovered from muscles		Comments
			Total	Average	
I	5	0	182,000 to 600,000	345,000	
II	12	50,000	0	0	
III	12	30,000	0	0	
IV,A	12	18,000	17, 41, 1; 9 rats, each 0	5†	Progeny of these larvae fed to groups IV,C,1, 2, and 3
IV,C,1	4	0	40,000 to 177,000	108,000	
IV,C,2	6	0	224,000 to 514,000	410,000	
IV,C,3	6	18,000	3, 0, 4, 2, 2, 7	3	
V,A	10	10,000	30 to 1,105	455†	Progeny of these larvae fed to groups V,C,1 and 2
V,C,1	7*	0	76,000 to 359,000	189,000	
V,C,2	4	10,000	43, 2, 4, 1	12†	Progeny of these larvae fed to groups V,E,1 and 2
V,E,1	6	0	279,000 to 464,000	339,800	
V,E,2	5	10,000	4, 0, 0, 170, 0	35	

* All rats were each fed 5,000 larvae except those of group V,C,1 which were each fed 4,000 larvae.

† Yield of larvae was built up by passing all larvae recovered from muscles through successive rats.

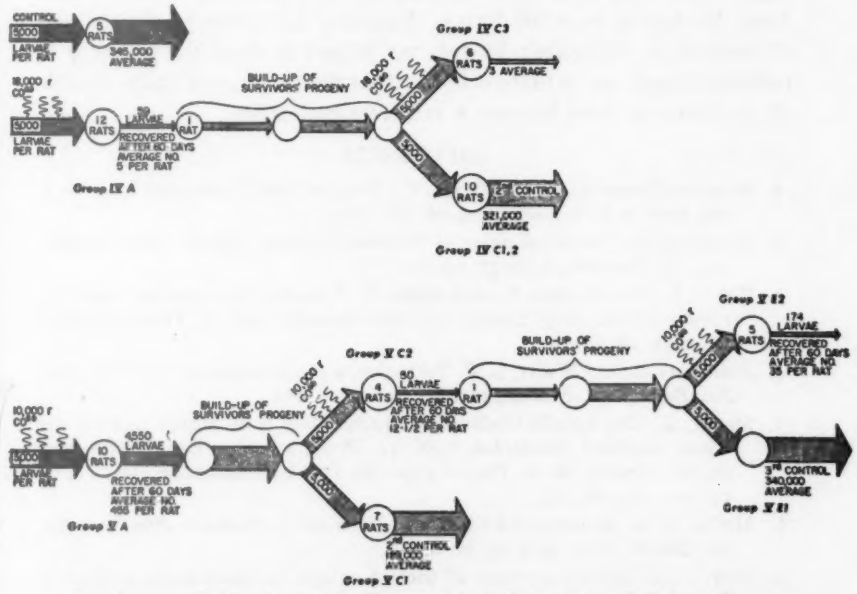
and the muscles digested for recovery of larvae. If the total number of larvae recovered from a group of rats was less than 5,000 for subsequent testing, the larvae recovered were fed to additional series of rats in order to build up their number. When a sufficient number were obtained, the larvae were exposed to various amounts of cobalt-60 and fed to uninfected rats. Upon sacrificing, the muscle larvae recovered were compared with those from the first groups of rats fed irradiated larvae.

As a check upon the number of larvae recovered from the muscles, microscopic sections were made of the tongue, diaphragm, and skeletal muscles of all rats at time of death or sacrifice.

Results

Reference to Table VII shows that infection of 5 control rats each fed 5,000 non-irradiated larvae resulted in an average infection of the muscles with 345,000 larvae. Doses of 50,000 and 30,000 r. cobalt-60 totally prevented reproduction, as determined by failure to recover any larvae from the muscles of any of 12 rats of group II or of group III.

Digestion of the muscles of 12 rats (group IV,A), each given an infective dose of 5,000 larvae exposed to 18,000 r. cobalt-60, resulted in a total recovery of only 59 larvae (an average of 5 larvae). These larvae were fed to rats, successively, to obtain a sufficient number for further tests. Some of these larvae were fed in the non-irradiated state



Text-figure 3. Study of possible development of radiation-resistance in succeeding generations of trichina larvae (Table VII).

to other controls (groups IV,C,1 and IV,C,2) while others were again irradiated with 18,000 r. cobalt-60 (group IV,C,3) and fed to each of 6 rats. The total yield from the ensuing infection in these 6 rats was

only 18 (average of 3 larvae). This finding did not point to the development of radioresistance.

Similar findings were obtained on feeding larvae exposed to 10,000 r. cobalt-60. Rats of group V,A fed larvae exposed to this amount of radiation yielded an average of 455 larvae on digestion of their muscles; rats of group V,C,2 fed a subsequent generation of these larvae exposed to the same amount of radiation yielded not an increased number of parasitic forms but actually a reduced number (average of 12). When the experiment was repeated by feeding the progeny of these to rats of group V,E,2, an average of only 35 larvae was obtained.

Text-figure 3 is a flow-chart summarizing these findings.

Summary and Conclusion

Larvae of *Trichinella spiralis* were exposed to cobalt-60 in a dose (10,000 r.) that causes sexual sterilization of most of the developing adult worms and in a dose (18,000 r.) that prevents most of them from developing to adult forms. Exposure of larvae to these doses of irradiation with cobalt-60 does not induce in them the property of radioresistance, nor is there evidence that the progeny of those capable of reproduction have become a radioresistant strain.

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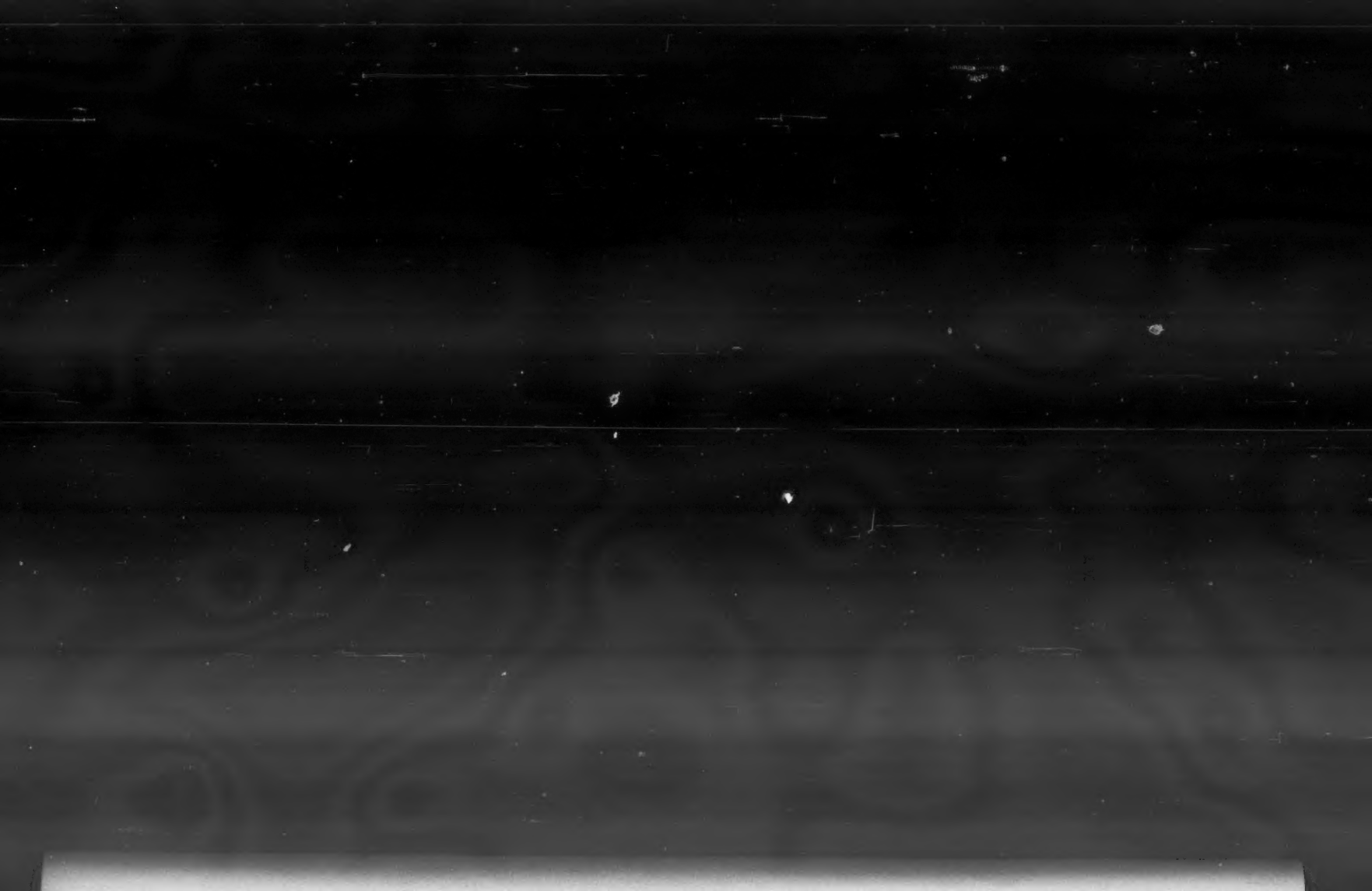
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Male adult firmly anchored in a villus. Most of the anterior portion (below) and the terminal portion with its copulatory appendages (above) lie between adjacent villi. Seminal vesicle is distended with sperms. Section of small intestine of white rat 33 hours after feeding excysted larvae. × 460.

FIG. 2. Anterior portion of female adult within mucosa. Vulva (v) opens on the concave surface of the lower portion of the right arm of the loop, and sperms are shown in the forepart of the uterus (ut) in the left upper portion of the field. Remaining portion of worm, not shown in the section, apparently lies outside of villus. 33 hours. × 440.



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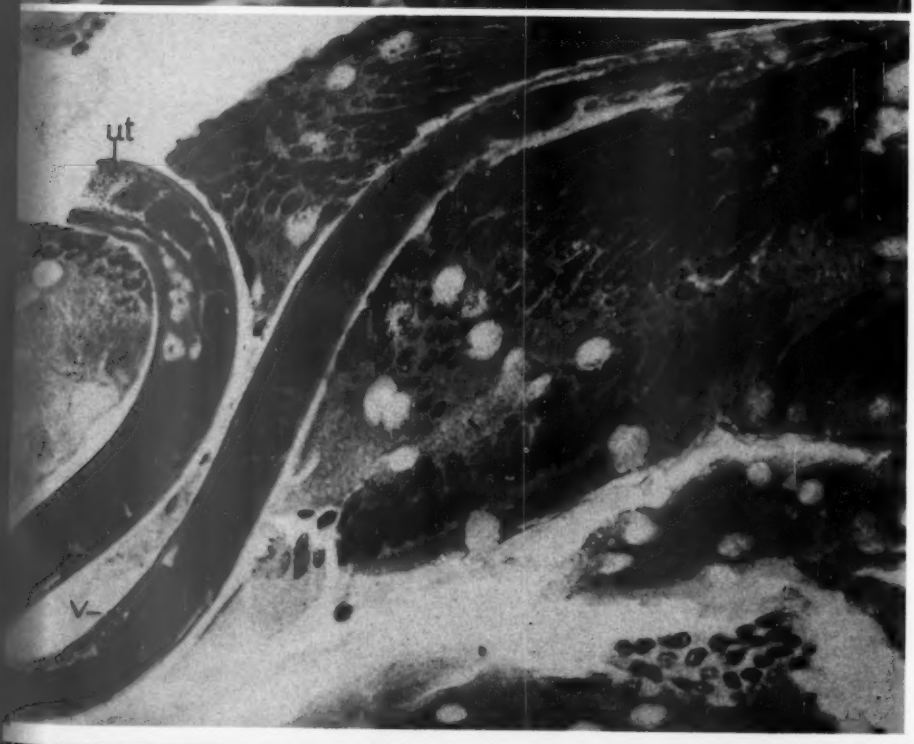

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FIG. 3. Uterus of female adult, containing sperms. 33 hours. × 460.

FIG. 4. Portion of adult female showing part of cell body (above) and of ovary (below) and the uterus between these structures. There is an early accumulation of sperms in the seminal receptacle. 33 hours. × 385.

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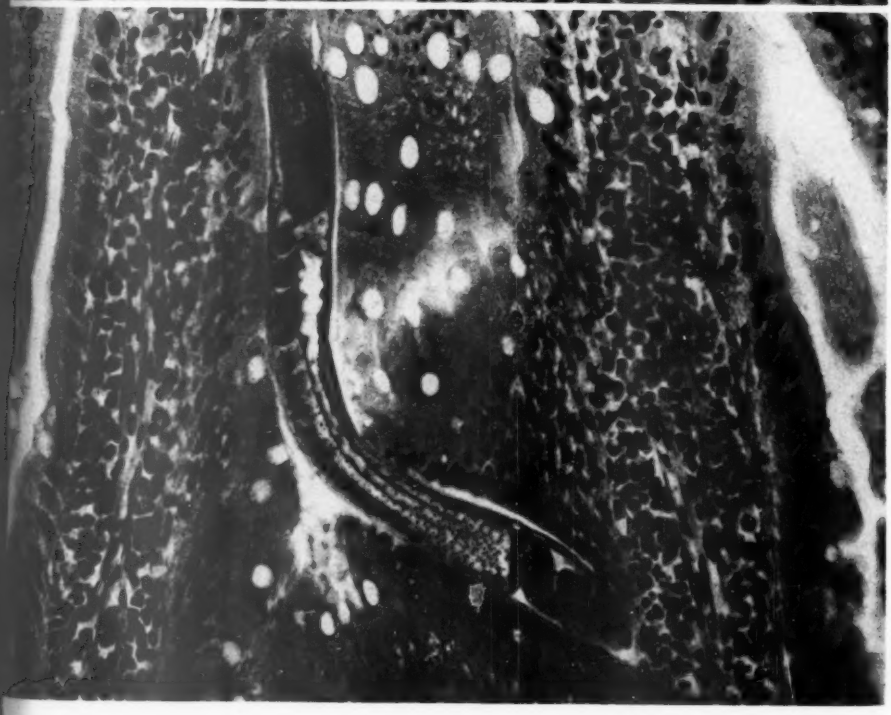

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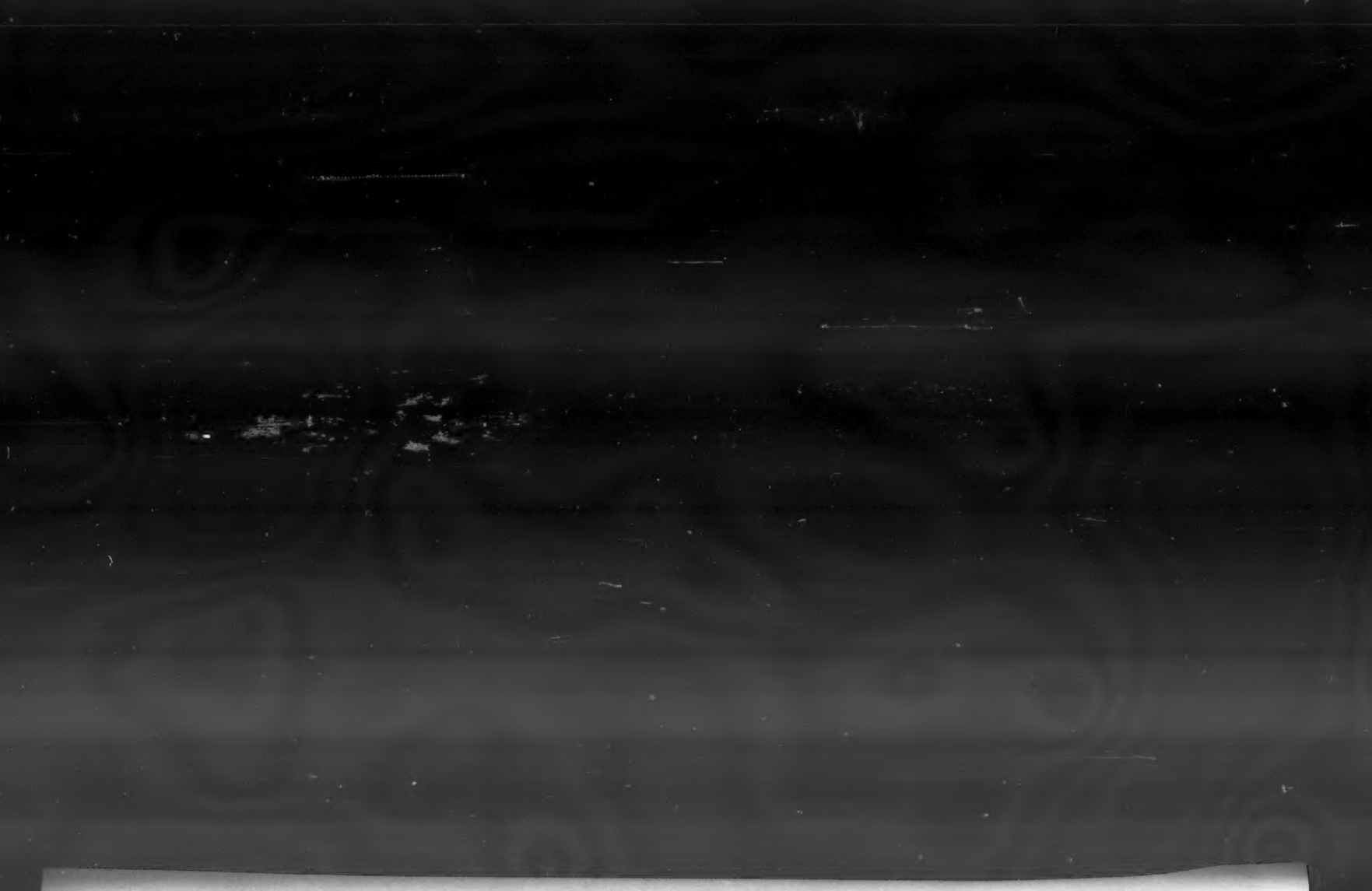
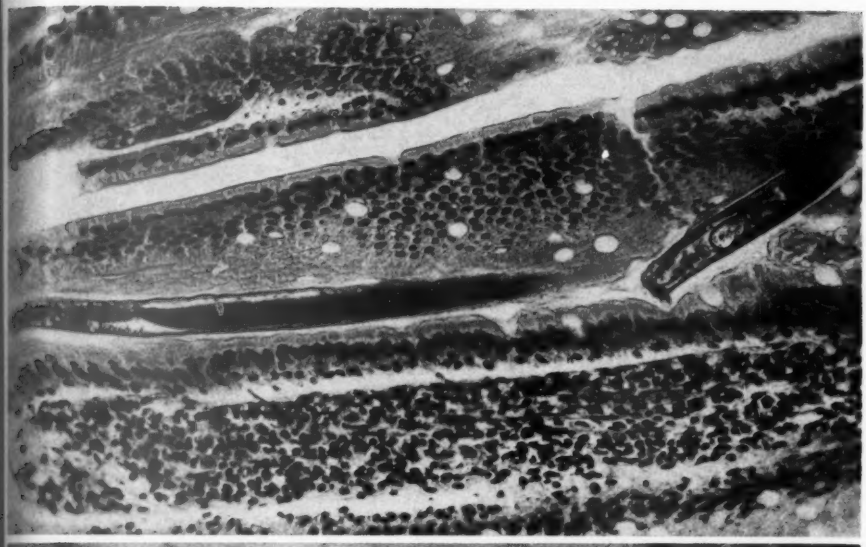
FIG. 5. At right, posterior portion of an adult male worm lies between adjacent villi. Of note are the copulatory appendages, and sperms within seminal vesicle. Remaining portion of the worm, not shown in the figure, is directed upward into a villus. At left, anterior portion of an adult female lies between adjacent villi. The vulva of the worm (not shown in the figure) apparently lies in close proximity to the copulatory appendages of the male. 33 hours. × 280.

FIG. 6. Larva of *Trichinella spiralis* recovered from the blood of a white rat 118 hours after infection. Blood was hemolyzed with 3 per cent acetic acid, and sediment was stained with Wright's blood stain. × 375.





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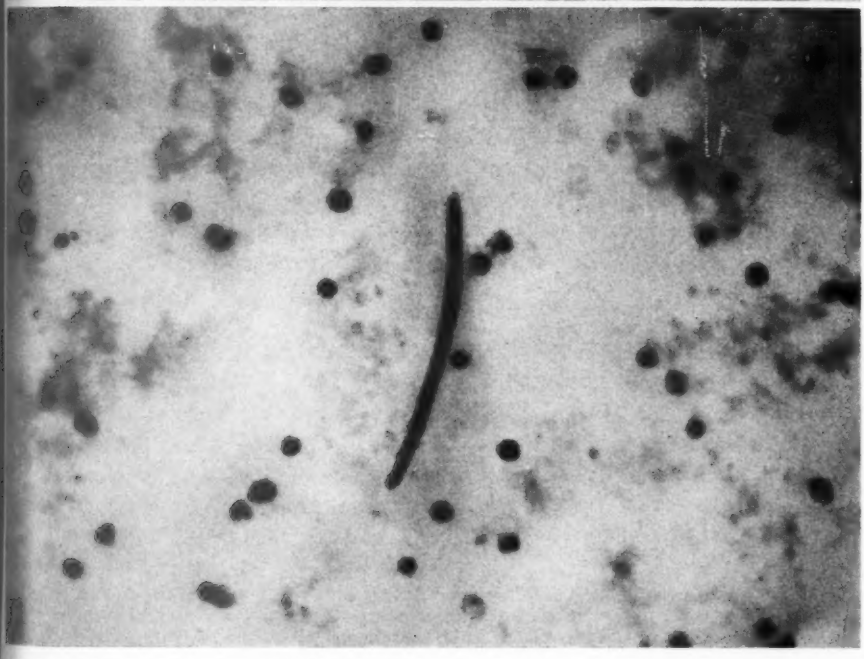

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EXPERIMENTAL ATHEROSCLEROSIS IN THE RAT *

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The rat has proved refractory to the development of experimental atherosclerosis. Page and Brown[1] were able to maintain very high serum-cholesterol levels for prolonged periods by feeding high-cholesterol, high-cholic-acid diets to hypothyroid rats. Although the aortas and coronary arteries of their animals showed lipid deposition, they found no foam cells or cellular proliferation and concluded that the vessels of the rat did not respond to the presence of lipid. Several other groups, however, have claimed success in producing lesions simulating atherosclerosis in this species. In 1952, Wissler *et al.*[2] reported moderate hypercholesterolemia and coronary atheromas in old rats fed a high-fat diet for many months. Increasing the choline content of the diet increased the incidence of lesions. In the same year Hartroft *et al.*[3] reported the production of lipid deposits in the coronary arteries of rats on choline-deficient diets. More recently Malinow *et al.*[4] have reported atheromatous lesions following the forced feeding of vegetable oil in the presence of unilateral cellophane perinephritis. Also in 1952 one of us[5] reported the production of early lesions in rats by the repeated intravenous injection of lipoproteins from the serum of cholesterol-fed rabbits. Lesions are readily produced in rabbits by this method,[6] but the occurrence of spontaneous lesions[7] is a complicating factor in acute experiments. The purpose of the present paper is to report in more detail the effects of injecting serum lipoproteins into rats and to illustrate the tissue response of rats to hypercholesterolemia.

METHODS

Young rabbits were rendered hypercholesterolemic by feeding a 1 per cent cholesterol diet. The cholesterol was dissolved in ether and the solution distributed over commercial rabbit pellets. After 6 to 10 weeks the animals were exsanguinated. Ten gm. of sodium chloride were added to each 100 ml. of serum. This increased the saline density to about 1.065. The serum was then centrifuged for 18 hours in the 30 rotor of the Spinco model L ultracentrifuge at 78,000 G. Under these

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conditions all the low-density lipoproteins (Gofman's Sf classes[8]) rose to the surface, where they appeared as a butter-like mass. This material re-emulsified readily in physiologic saline solution, against which it was then dialyzed to remove any excess salt. No attempt was made to keep the emulsions sterile. In the chronic experiments fresh emulsions were prepared weekly.

The experimental animals were male Sprague-Dawley rats weighing about 200 gm. Injections were made into a tail vein. The volume injected varied from 1 to 4 ml. At the termination of an experiment the animals were exsanguinated from the aorta under sodium pentobarbital anesthesia. The heart and thoracic aorta were removed en bloc and irrigated with physiologic saline solution. They were then fixed in formalin, embedded in gelatin, sectioned with the freezing microtome, and stained with oil red O and hematoxylin.

The following lipid analyses were made both of the emulsions and of the recipients' serum: free and total cholesterol,[9] phospholipid,[10] and total fat.[11] From these, neutral fat was determined by difference.

Other rats were offered the following diet: vitamin-test casein, 19 per cent; dextrin, 31 per cent; lard, 30 per cent; wheat bran, 6 per cent; salt mixture, 6 per cent; complete vitamin powder, 5 per cent; cholic acid, 2 per cent; oleic acid, 1 per cent; cholesterol, 0.3 per cent, and 6-propyl-2-thiouracil, 0.065 per cent. Fat-soluble vitamins were supplied as Natola,* 10 drops per kg. of diet. The animals were housed in individual cages and offered the diet and water ad libitum.

Results

Because the lipoprotein emulsions represented pools of several donor rabbits, they were relatively constant in chemical composition. The following analysis, expressed as mg. per 100 ml., is typical: total cholesterol, 6,860; free cholesterol, 1,740; phospholipid, 2,440, and neutral fat, 980. The average cholesterol-phospholipid ratio was 2.8. Some preparations were more concentrated.

The greater the injected dose, expressed as mg. of total cholesterol per kg. of body weight, the higher was the serum cholesterol level 24 hours later. Thus, rats receiving a dose of 2,650 mg. had serum levels of about 2,650 mg. per 100 ml.; animals receiving 2,000 mg. had serum levels of about 1,250, and those receiving 650 mg. had levels of 180. The serum of normal rats contains about 50 mg. of cholesterol and 100 mg. of phospholipid per 100 ml. After subtracting these values, the cholesterol-phospholipid ratio in several rats receiving the same

* Parke, Davis & Co., Detroit, Michigan.

emulsion dropped from the immediate post-injection value of 2.9 to about 2.5 at 7 hours and 1.6 at 24 hours.

Control rats have never shown stainable lipid within the intima or media of the aorta or coronary arteries, or in the endocardium. Animals receiving lipoproteins in doses of 1,000 mg. total cholesterol per kg. uniformly show focal deposits of sudanophilic material at 24 hours. These areas are characterized by small, discrete granules of lipid within the cytoplasm of endothelial cells and, in the endocardium, in cells immediately beneath the endothelium. In the aorta there may also be very fine sudanophilic particles, apparently extracellular, along the inner surface of the innermost elastic lamella. In the aorta these deposits appear to have no sites of predilection, such as about the mouths of the great vessels. In the heart they appear on the aortic cusps and mitral leaflets. The endothelium of the coronary arteries is only rarely involved. With larger doses these changes have been observed as early as 4 hours after injection.

More advanced lesions may be produced either by continuous intravenous infusion or by repeated daily injections. Two rats received a constant infusion at a rate slightly in excess of 1 ml. per hour for 24 hours, at which time their serum cholesterol levels were 11,700 and 10,700 mg. per 100 ml., respectively. Endothelial and subendothelial cells of the endocardium had assumed the appearance of foam cells and there was evidence of their focal proliferation.

Figure 1 shows a section of aorta of a rat which had received five daily injections. At one end of the photomicrograph the endothelial cells contain lipid granules; at the other end there is a collection of sudanophilic material which appears to have accumulated along the inner surface of the most internal elastic lamella. There is no evidence of tissue reaction.

Sixteen animals received daily intravenous injections for periods varying from 22 to 30 days. All animals gained weight during that time. Figures 2 and 4 illustrate lesions in a rat killed 24 hours after receiving 28 daily injections. Figure 2 shows a lesion on the endocardial surface of the left ventricle. The endocardium is thickened by masses of foam cells. Figure 4 shows a coronary artery at its origin from the aorta. Two focal accumulations of foam cells are protruding into the lumen of the vessel. Schultz stains revealed cholesterol in these lesions. Neither necrosis nor inflammatory response was seen. The sections originally showed no anisotropism, but doubly refractile crystals became abundant with the passage of time. Other coronary lesions have been limited to infiltration of the intima and media with-

out cellular reaction. In the aorta this infiltration has been limited to the intima.

Some of the rats receiving repeated injections were permitted to survive for periods up to 3 months following the last injection. The amount of sudanophilic material in the endocardium gradually diminished although even at 3 months some was present both intracellularly and extracellularly. Anisotropic crystals became a prominent feature of the lesions. Eventually they were embedded in fibrous tissue, although at no time was there evidence of necrosis or active inflammation.

Examination of other organs of rats killed 24 hours after 28 daily injections showed slight enlargement of the adrenal glands, and microscopic, but not gross, fatty infiltration of the liver. Sudanophilic material was present within a few cells of the splenic corpuscles and within the renal glomeruli. The tubules, however, were practically free of fat. The pulmonary arteries occasionally showed some sudanophilia. Other vessels were not examined.

No quantitative or qualitative differences were observed in the tissue response of 2 female and 2 male rats receiving repeated injections of the same material. Old males were compared with young males, the dose being constant on a weight basis. The older animals appeared to have more extensive lesions than the younger. Old animals were not used routinely in this study because greater difficulty was encountered in injecting their tail veins.

Twenty-four hours following intraperitoneal injection the serum cholesterol level was higher than following an intravenous injection of the same dose. Foam-cellular lesions were produced following repeated intraperitoneal injections, but peritonitis was a complicating factor.

Three rats received 28 daily intravenous injections of a synthetic cholesterol emulsion in a dose of 200 mg. per kg. per day. Twenty-four hours after the last injection serum cholesterol levels were in the normal range and no sudanophilic material could be found in the endocardium or arterial intima. In this respect the rat differs from the rabbit, which converts injected cholesterol into lipoproteins. The emulsions could not be concentrated to permit larger doses.

In order to compare these lesions with those observed in hypothyroid rats fed cholesterol and cholic acid, 5 rats were placed on the diet described. All animals slowly lost weight over a period of 22 weeks, at the end of which time they were killed. Serum cholesterol levels at that time averaged 944 mg. (776 to 1,370) per 100 ml. The cholesterol-phospholipid ratio averaged 2.0 (1.7 to 2.2). All animals showed

vascular sudanophilic deposits. In 2 cases there were foci of foam cell proliferation in the endocardium. They appeared identical to those produced by injecting lipoproteins. Figure 3 illustrates such a lesion on a mitral leaflet. Lesions in the coronary arteries were characterized by foci of massive infiltration of the media with sudanophilic, anisotropic material but without tissue reaction. The infiltration occasionally was sufficient to reduce the lumen of the vessel substantially. Lesions in the aorta were, like those following lipoprotein injections, without cellular reaction.

Discussion

The evidence presented here demonstrates that parts of the vascular system of the rat are capable of responding to hypercholesterolemia in an atheromatous manner. Figures 2 and 3 from injected and dietary rats, respectively, show masses of foam cells thickening the endocardium. This has been a frequent finding. Figure 4 shows the same process in a coronary artery near its ostium. This remains a unique example in our material. Usually, in the smaller branches, there are seen only foci of massive infiltration of the wall with sudanophilic, anisotropic material, but without cellular reaction. An illustration in a recent paper by Wissler *et al.*[12] apparently shows foam-cellular proliferation in a coronary artery.

In our experience, with the one exception illustrated, tissue reaction to the presence of lipid has occurred only in the endocardium. These proliferating lesions may be lost when frozen sections are cut from unembedded hearts. This may explain the failure of Page and Brown[1] to observe them.

To date, apparently no one has succeeded in producing atheromatous lesions in the aorta of the rat. The lesions illustrated in the paper by Hartroft *et al.*[3] from choline-deficient rats represent primarily medial degeneration. It is possible that this immunity of the aorta of the rat has an anatomical basis. The intima consists of little more than endothelium. We have seen masses of lipid granules between the innermost elastic lamella and the endothelium, so that the latter protrudes into the lumen, and yet there has been no evidence of cellular reaction.

The data further suggest that the development of atheromatous lesions in the endocardium of the rat depends primarily on maintaining a sufficient concentration in the blood of cholesterol-bearing lipoproteins for a sufficient period. This does not deny that factors such as hypertension and local vascular injury may accentuate the atherosclerotic process.

SUMMARY

Focal atheroma-like lesions have been produced in the rat by intravenous or intraperitoneal injection of lipoproteins from the serum of cholesterol-fed rabbits. The lesions are characterized by groups of foam cells in the endocardium and intima and later by masses of anisotropic crystals embedded in fibrous tissue. Similar lesions have appeared in hypothyroid rats fed a diet relatively high in cholesterol and cholic acid. The lesions occur most frequently on the aortic and mitral valves and in the endocardium of the left ventricle. One such lesion has been found in a coronary artery near its ostium. Coronary arteries usually show only massive infiltration of the wall without tissue reaction. The aortic intima may also show heavy deposits of lipid without tissue reaction. It is concluded that the arteries of the rat are relatively immune to those lipoproteins which in the rabbit are highly atherogenic.

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[*Illustrations follow*]

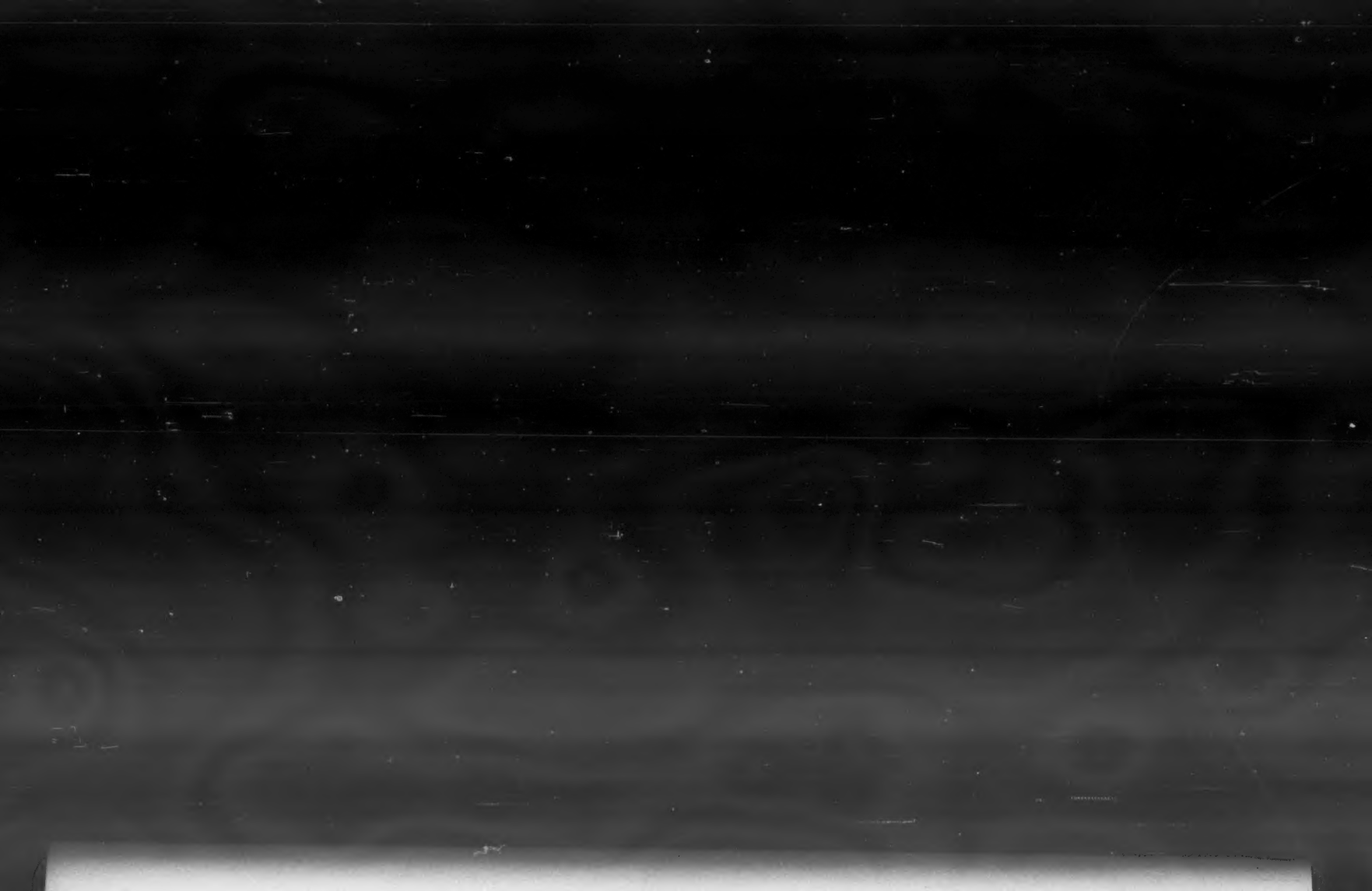
LEGENDS FOR FIGURES

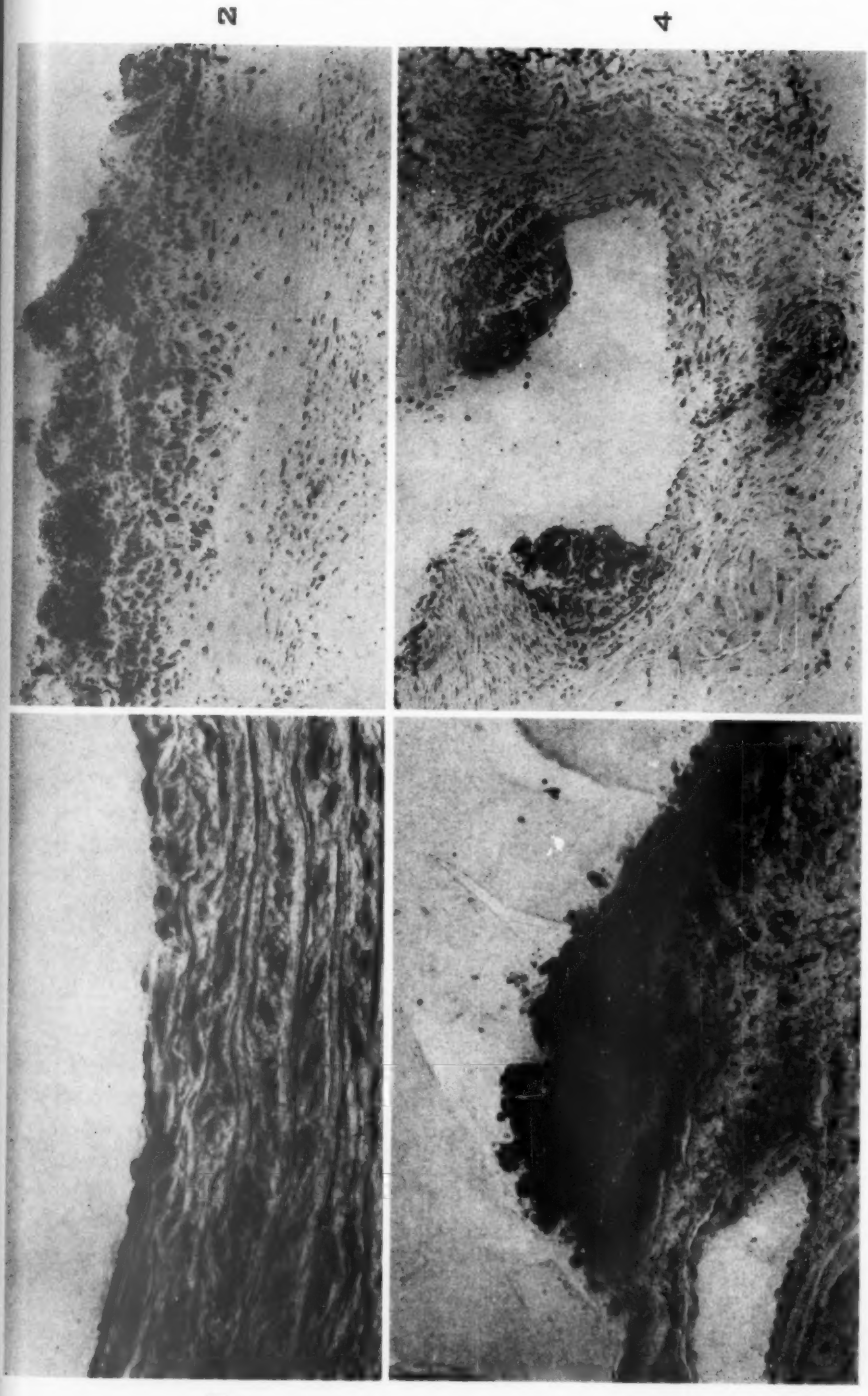
FIG. 1. Aorta of a rat which received five daily intravenous injections of lipoproteins from the serum of cholesterol-fed rabbits. Sudanophilic material is apparent both within endothelial cells and extracellularly between endothelium and innermost elastic lamella. × 210.

FIG. 2. Endocardium of left ventricle of a rat which received twenty-eight daily injections of lipoprotein. The endocardium is thickened by masses of foam cells. × 140.

FIG. 3. Section of base of mitral leaflet from a rat with dietary hypercholesterolemia. The endocardium is greatly thickened by foam cells. Under polarized light this lesion contained doubly refractile crystals. × 170.

FIG. 4. Section through ostium of a coronary artery of the same rat from which Figure 2 was secured. Two masses of foam cells protrude into the lumen of the vessel. Sudanophilic material is seen also within the aortic intima and within the media of a small coronary branch, but without cellular reaction. × 140.

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